The Development and Application of Chromatographic Techniques in the Characterisation of Artists' Media

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A thesis submitted in partial fulfilment

of the requirements of the

University of Northumbria at Newcastle

for the degree of Doctor of Philosophy.

In collaboration with the Tate Gallery, London

August 1997

To Mum and Helen,

for their

unfailing patience, support and love.

ABSTRACT

The aim of this project was the development and application of chromatographic techniques for the characterisation of proteinaceous and natural gum materials used as binders, adhesives *et cetera* by artists.

Proteinaceous materials (animal glue, casein and egg proteins) were hydrolysed under acid conditions prior to derivatisation with 9-fluorenylmethyl chloroformate: the amino acid content of the samples was then determined by reverse phase high performance liquid chromatography. Sixteen amino acids were monitored, their relative percentages providing a means of differentiating between the proteinaceous media types.

Natural gum materials were silylated with hexamethyldisilazane after acid hydrolysis, then their sugar components were separated and identified by gas chromatography-mass spectrometry, using selected ion monitoring for maximum sensitivity. A sugar composition 'fingerprint' was obtained for each standard gum material analysed, facilitating the characterisation of gum media from works of art.

Samples of suspected proteinaceous and gum media were removed from works of art from the Tate Gallery's collection, focusing on 18th and 19th century British works, particularly those of William Blake, J.M.W. Turner and Dante Gabriel Rossetti. The characterisation of media samples from such works provided valuable information regarding the nature of media preferred by artists of this period, additives to gum media and, perhaps most importantly, the relationship between binding media and support and their effects on the condition of ageing paintings.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people and organisations, without whose contributions this project would not have been possible:

Dr. Joyce Townsend, of the Tate Gallery's Conservation Department, for her invaluable advice and assistance throughout;

Drs. Steve Hitchen and Brian Singer, for their guidance and supervision;

Mr. Ed Ludkin and Miss Angela Pocklington, for their technical advice and support;

Dr. Bill Tomlinson, for his assistance with the chemometric studies;

The Higher Education Funding Council for England (HEFCE) and University of Northumbria at Newcastle for financial support;

Tate Publications for their kind permission to reproduce illustrations of works from the Tate Gallery's collection; and finally,

My family and friends who were always there when I needed them.

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CHAPTER 1

Introduction

1.1 Project Aims

The artist's selection of a binding medium was/is influenced, not only by the types of pigment and support used, but by historical factors like their location and the period of the piece: fashion and scientific progress have ensured that different materials have enjoyed periodic popularity.

There is a need for the development of methods for the analysis of samples from works of art, in order to meet the specific requirements of the conservator. A detailed awareness of the constituents of paint layers from easel paintings, for example, provides the conservator with the background information required to facilitate the design of the optimum safe conservation/restoration treatment plan, taking into account the nature of the original materials used.

From a practical aspect, specific knowledge of the nature of the media in particular works may offer some indication as to why some paintings are in better condition than others of a similar age. This type of information also enables the art historian to come to an educated and informed conclusion regarding the age and potential origin of an unknown work. In addition, it is

feasible that a counterfeit work may be revealed, if the artist has been careless enough to use pigments or binders that are historically incorrect.

The ultimate aim of the project is the development of chromatographic methods for the analysis of artists' materials, primarily gum and proteinaceous binding media, in order that art historians and conservators can understand more about the materials and techniques employed by the artists in question. Proteinaceous binding materials will be characterised *via* amino acid analysis following the development of a suitable reversed phase high performance liquid chromatographic (RP-HPLC) method, whilst gas chromatographic/gas chromatographic-mass spectrometric (GC/GC-MS) methods will be developed and employed for the analysis and identification of the sugar components of gum media.

The project focuses on the correlation between the media used and the condition of the works studied, along with the choice of media by a particular artist. The study involves determining the way in which artists chose their media (*i.e.* did artists tend to use just one particular medium throughout their career or did they use a selection of single and/or mixed media at various stages?) and relating this information to the condition of their works. By comparing condition with medium, plus medium with artist, it is hoped that valuable information on the effects of binding media may be obtained.

Samples from a selection of works which span the career of each artist under consideration will be studied, in order that any variations in the choice of binding media may be determined. Documentary evidence, detailing information on the condition of the work, the area of the work which was sampled, the specific nature of any conservation problems along with the date

of the work and the artist's identity, must be supplied with each sample submitted for analysis. The condition of the works has been classified by the following criteria: the extent of any flaking/cracking, the amount and nature of any retouching/consolidation work already performed plus the degree of light sensitivity and discolouration.

It is anticipated that, using the methods developed in the course of the research, a catalogue of information on works by European artists, such as William Blake, may be compiled, providing unique information concerning the composition of the paint media which is currently unavailable, due to the lack of investigation into the applicability of chromatographic techniques for these purposes.

1.2 Artists' Binding Media

Since man first learned to paint artists have used a diverse array of binding media for their pigments, ranging from natural gums and oils to proteinaceous materials like egg (*glair* and *tempera*), milk (*casein*) and collagen glues made from animal skins and skeletons, for example.

Chemically, oils and fats are the glycerol esters of aliphatic acids, commonly those of the 18-carbon series (*e.g.* stearic acid [$C_{18}H_{36}O_2$], oleic acid [$C_{18}H_{34}O_2$] and linolenic acid [$C_{18}H_{32}O_2$]):[1] oils tend to be liquid at room temperature, whilst fats are solid or semi-solid and greasy to the touch. The physical and chemical properties of individual oils and fats are determined by the type and proportion of each of the aliphatic acids contained in their constituent triglycerides and the presence of any triple ester functions and reactive double bonds. The presence of di- and tri-unsaturated fatty acids

in an oil's triglyceride components dictates its ability to 'dry', *i.e.* polymerise giving rise to a semi-solid. This polymerisation process is thought to involve the addition of an oxygen molecule at each double bond, but it is not only the level of unsaturation which determines drying ability – the actual type of unsaturation plays a major role: perilla oil is more unsaturated than linseed oil, but gives an irregular film, possibly due to the higher levels of linolenic acid present.[1] The drying oils most widely used in Western European art are linseed (obtained from the seeds of the flax), walnut and poppy: the time when they were first used for painting purposes is not known, though it is generally accepted that by the turn of the 15th century, oil painting had become widespread over the previous two hundred years.[2] Throughout the world, various drying oils are produced by indigenous trees and plants, many having a long history of local use. The use of hempseed and perilla oils is documented in a 9th century Japanese manuscript[3] and chia oil, from the seed of *Salvia hispanica*, was used in Mexico during the Spanish colonial period.[4] In the 20th century some exotic oils (tung, perilla and safflower, for example) have moved to large scale production for commercial use.

The materials referred to as waxes do not actually form a chemically homogenous group, but chemists describe a wax as a material comprised of long chain hydrocarbons, alcohols, esters and acids in varying amounts. Unlike fats, the esters present in waxes are derivatives of the long chain acids and alcohols and are saturated. The name *wax* is commonly applied to materials which possess wax-like characteristics, *i.e.* are translucent solids with low melting points and a 'waxy' texture. The three main origins of waxes are insects/animals (*e.g.* beeswax, shellac, lanolin and spermaceti

wax), plants (*e.g.* carnauba, ouricuri, candelilla and jojoba) and fossils/minerals (*e.g.* ceresine, peat and paraffin).[5] Waxes, predominantly beeswax, have been widely used for many centuries: the ancient Egyptians used beeswax as an adhesive or a surface coating, whilst the Greeks and Romans probably used it as a waterproof coating for painted walls.[5] In the 18th and 19th centuries, beeswax has been used as an ingredient in original painting media and waxes themselves have found many uses in modern conservation practices.

1.2.1 Proteinaceous Binding Materials

Collagen is the predominant proteinaceous material in animal skeletons (both skin and bone), representing one third of the total protein present in mammalian organisms.[6] Collagen production in the body is preceded by the production of *procollagen*, a much larger biosynthetic precursor molecule, which is then degraded by specific enzymes resulting in collagen. There are a number of different types of collagen, but they all consist of molecules which contain three polypeptide α-chains in a triple helix conformation. Each α-chain has an amino acid sequence which is mainly a repeating structure, with glycine as every third residue and either proline or hydroxyproline often preceding the glycine residues. The various types of collagen can be distinguished by the slight differences in the sequence and ratios of their constituent amino acids.

Animal and fish collagen glues are widely used as strong adhesives for wood, binders in the preparation of grounds, dressing for canvas and pigment

binders in decorative paints.[7] Preparation is relatively simple, involving the treatment of specific collagen-containing animal/fish tissues with hot water. When the leached solution cools, it forms a gelatinous mass: gelling occurs as a result of the partial decomposition of the tissue. If the extraction process is performed at a lower temperature (*e.g.* 80-90 °C) the gelatine solutions formed from the collagen are usually clear and light yellow in colour: pure forms of gelatine are usually referred to as 'size'. A glue which has been prepared at an elevated temperature is less pure, much more turbid and viscous and is considerably darker in colour.

Animal glues swell and soften when soaked in cold water, and drying restores their original properties. When gently heated with water, glues yield a viscous liquid, but lose strength with heating: however, all glues spoil with age, eventually losing their adhesive powers.

Fish glue and isinglass, the impure and pure gelatines obtained from fish bones/skins and bladders respectively, are readily soluble in water and can be purchased in both liquid and sheet form. Fish glues do not possess the adhesive strength of animal glues and tend to spoil more easily by bacterial decomposition.[8]

Animal glues are widely used in the preparation of painting grounds: gesso - an aqueous, white priming/ground material commonly used for the preparation of late medieval panels and other supports - contains chalk or gypsum, with glue as the binding medium. In medieval books, where a thick but flexible application of paint was required, a glue medium was preferred and the ancient Egyptians often mixed animal glue with gums, eggs and wax for decorative painting.[7]

Casein is a mixture of related phosphoproteins found in milk products. It is produced in mammary tissue from amino acids which have been supplied by the blood, contains all the common amino acid residues and is particularly rich in those deemed as essential, *e.g.* leucine.[9]

Albumins, the proteins present in eggs and living tissue, are water soluble, globular proteins which are rapidly denatured by heat, rendering them insoluble.[10] The adjacent amino acid clusters present in the protein are internally hydrogen-bonded, resulting in the protein's tight, ball-like configuration: polar, hydrophilic groups form the surface of the ball whilst any hydrophobic groups are tucked inside the ball.

Egg albumin and yolk have very few differences in their amino acid content, indeed it is the even proportions of essential amino acids which makes the egg such a good source of dietary protein. Eggs have a complex composition, but over 50 % of hens' egg albumin is a glycoprotein called ovalbumin and a further 15 % is conalbumin, also a glycoprotein. Egg yolk is protein rich, containing phosphovitin (a composite protein), α- and β-lipovitellins (lipoproteins containing phospholipids) and α-, β- and γ-livetins.[10]

Egg albumin (*glair*) and yolk (*tempera*) have found uses as pigment binders, temporary varnishes and sealants over primers or grounds in Western art from the 13th century: they have been used this century by a small but significant number of artists. Casein has similar uses to those of egg, but additionally provides one of the strongest natural adhesives known, much used by cabinet makers and joiners. There are numerous recipes for the

preparation of egg-based media containing ingredients as varied as linseed oil,[11] fig tree shoots[12] and vinegar,[13] but the preparation of casein is uncomplicated: skimmed milk is heated to 35 °C, then the mixture is acidified to pH 4.8 with hydrochloric acid. It is allowed to stand and the casein solids are separated from the supernatant liquid, then washed with hydrochloric acid (pH 4.8) - the casein prepared thus is a technically pure, slightly hygroscopic white powder.[9]

Ancient Hebrew texts mention the use of casein for house painting and decoration, whilst Michelangelo[14] allegedly added sour milk to his oil paints in order to produce highlight effects on walls: casein was also used as an adhesive and consolidant for oil paint by late 19th century conservators. Today, however, casein is less commonly used as a painting medium by artists, possibly since a casein film is inflexible and insoluble, thereby causing difficulties when handling or attempting alterations. Casein colours harden and crumble when stored, though the addition of glycerine keeps the paint moist, whilst increasing its water-solubility. The primary problem associated with a casein medium is its tendency to encourage mould growth.

1.2.2 Polysaccharide Gum Binding Materials

Gums are a group of non-crystalline, polysaccharide materials which can be found in vegetable matter and are often exuded when a plant is wounded. They are either water-soluble or water-dispersible compounds with complex composition, usually consisting of a number of sugars (*e.g.* galactose and mannose) plus the acids derived from them (*e.g.* galacturonic acid). Gums differ from gelatines and other proteinaceous materials which form

similar mucilaginous solutions in water, in that they contain virtually no nitrogen.[15]

Plant gums have been used for many centuries as the principal medium for watercolours, miniatures and manuscript illumination and as sizing materials; indeed there is documentary evidence to suggest that gum was used as a replacement for sundried oil as early as the 12th century.[16]

Gum arabic is most commonly used as a painting medium and has been utilised in this capacity for many thousands of years, being a particular favourite of the ancient Egyptians. The manuscripts of Jehan le Begue describe the preparation of a rose colour - powdered pigment was ground with an aqueous gum consisting of two thirds gum arabic and one third water.[17]

Gum arabic is produced by a number of species of *Acacia*, found growing in India, Africa and Australia, the most important being *Acacia senegal.* The gum is a natural exudate of the trees and is marketed in the form of solid lumps, which range from colourless to pale yellow. These lumps are brittle and breaking exposes the transparent interior, which is colourless in the finer grades of the gum. Church[18] recommends that senegal gum, which contains around 16 % water, should be the only variant used for artistic purposes: it should be transparent, leave no residue in a cold water solution and should give no colour with tincture of iodine.

For painting use, Church[18] suggests the following method of preparation: one part finely powdered gum arabic should be carefully mixed with two parts boiling, distilled water. This solution should be allowed to stand for no less than a day, after which time it should be decanted from any

remaining sediment into a glass stoppered jar. Camphor, eugenol or β-naphthol can be added as a preservative and the addition of a fatty oil will emulsify the gum solution - glycerine (<5 % in solution) may be used in order to increase flexibility.[19]

Gum tragacanth, known since classical times, has been used as a medium for painting on linen, though its primary use is as a binder in the manufacture of pastel crayons.[19] The gum is produced by a leguminous shrub belonging to the *Astragalus* species, which grows in parts of Asia and the East, particularly Iran. Collection is more difficult than for gum arabic, which leads to higher costs and this may be one of the reasons that gum tragacanth is used far less often.

Gum tragacanth is not entirely water soluble. It largely consists of bassorin, a mucilaginous substance which only swells in cold water, with a small amount of soluble tragacanthin. Gum tragacanth contains between 12 and 15 % water and may be prepared for use by wetting the finely powdered gum with alcohol prior to shaking with water. The gum, of which only 3 % dissolves, swells to form a mucilaginous mass which should then be strained through a cloth: it is therefore difficult to attain a uniform consistency. Gum tragacanth should be applied thinly when painting and the work should be left for some time before varnishing.

The gums obtained from trees of the *Prunus* species, particularly cherry, have been used as painting media in European works for many centuries.[20,21] Like gum tragacanth, cherry gum swells in water, but up to 10 % of the gum is actually soluble material. The solution should be strained through cloth and may be emulsified with balsams or fatty oils. Cherry gum

gives a transparency to colour and an enamel-like effect is achieved by mixing the gum with egg or casein emulsions.[22] However, if the gum is used alone or applied as a thin glaze, it is easily chipped.

Honey has long been used as an additive in aqueous media (like gum arabic, size or glair) and was a common ingredient in moist water colours until quite recently.[23] Since honey retains a certain amount of water, around 20 %, it prevents the extreme drying which results in the brittleness of these materials. However, since the 19th century glycerine has more commonly been used for this purpose.[19]

Other plant gums, including carob or locust bean, tamarind, cholla, plus plant resins like myrrh and olibanum, have found uses in an artistic context either for painting or paper making.[20] Karaya gum, the dried exudate of the native Indian tree *Sterticula urens*, has been used as a cheaper substitute to gum tragacanth; alternative names include Indian tragacanth, Indian gum and Sterticula gum.[24] It is thought that the Egyptians used carob gum for binding mummy wrappings and tamarind gum was the principal paint medium for Indian miniatures and murals.

CHAPTER 2

Background Chemistry

2.1 The Analysis of Paint Media

The analyses of oil-based media are well documented,[25-28] but any information on the nature of other binding media has primarily been obtained *via* microscopic staining methods or crude solubility tests.[23,29-33] Existing methods of analysis have provided the basis for separating the general categories of binding media (oil, gum and protein) by qualitative means - differential staining of cross-sections can distinguish between oil and protein layers, for example.[29] Confirmatory staining tests require the use of mixtures of alcohol and neutral iron chloride, or sodium hydroxide with copper sulphate (Fehling's solution).[30,23]

An advantage of such micro-analytical techniques is their applicability *in situ* but the results can be misleading, with false positive and negative results being observed. For this reason, these techniques are best used in conjunction with other analytical methods.

These simple early qualitative techniques, including paper and thin layer chromatography,[31-35] are sufficient where only the general category of the binding medium is needed or, for example, where the binder may contain unique constituents, such as hydroxyproline in gelatine. However, where the amino acid or sugar composition of a proteinaceous material or gum is insufficiently distinctive, such as in the cases of egg and milk proteins for example, only a quantitative technique such as chromatography will enable differentiation between similar binding media.

2.2 Basic Introduction to Chromatographic Analyses

Chromatography is the generic name for a series of closely related techniques designed for the separation of components of a mixture, based on the partition of the components between two phases, one mobile and one stationary.

The stationary phase may be solid particles (often silica or alumina) with an unmodified or chemically modified surface, or a liquid supported on an inert solid, and usually covers a large surface area. The stationary phase may be packed into a column, *e.g.* liquid chromatography; spread onto a sheet of an inert support material (glass, plastic or aluminium foil), *e.g.* thin layer chromatography; or coated on the inner surface of a tube, *e.g.* capillary gas chromatography.

The mobile phase may be a gas or a liquid. Gas chromatography (GC) uses a carrier gas (commonly helium, nitrogen or hydrogen) to carry the analtyes through the column, which may be a fused-silica capillary column or one which is packed with the stationary phase material. GC is used for the separation of volatile compounds, *i.e.* those with an adequate vapour pressure within the normal operating range of the gas chromatograph itself (usually up to 350 °C).

Analysis of less volatile and more polar samples is achieved using a technique with a liquid mobile phase. Here, the stationary phase most commonly consists of porous particles with a chemically bonded monolayer which functions as the stationary phase. This technique of analytical liquid chromatography is usually referred to as high performance liquid

chromatography (HPLC), the most common type used being reversed phase (RP-HPLC).

In a reversed phase (RP) chromatographic system the mobile phase is more polar than the stationary phase. In general, the mobile phase is aqueous and the stationary phase is porous silica to which a variety of alkyl groups have been bonded *via* the silanol sites: for example, an ODS or C18 column has octadecyl groups bonded to the silica's silanol sites.

Retention and, therefore, separation in RP-HPLC occurs due to non-specific hydrophobic interactions of the solute with the stationary phase balanced with interactions with the mobile phase. The fact that almost every molecule has hydrophobic areas in its structure, which are capable of interacting with the stationary phase, leads to the wide application of RP-HPLC.

The general principle of the separation is the same, regardless of the technique being used. Solute molecules have varying degrees of affinity for the stationary and mobile phases, hence they partition themselves between the two phases. The system is optimised - too great an interaction with the stationary phase leads to long analysis times and poorer sensitivity with later eluting peaks, whilst insufficient interaction with the stationary phase leads to poorer separation.

The order in which the sample components are eluted from the column is dependent on the distribution of the analyte compound between the mobile and stationary phases. In GC the elution is most often controlled by temperature, whereas in RP-HPLC the order of elution reflects the

hydrophobicity of the analyte - the less polar the analyte, the greater its affinity for the stationary phase and hence the later it will be eluted.

2.3 Analysis of Proteinaceous Media

Quantitative amino acid analysis by means of ion exchange chromatography of protein hydrolysates was introduced by Moore and Stein in 1954.[36] A sample of hydrolysed protein was applied to a column of sulphonic acid resin (pH 3) and an eluent with increasing pH and salt content was pumped through the column. The eluted amino acids, 18 in total, were detected by their absorbancies produced with ninhydrin reagent (postcolumn derivatisation). The required sample size was relatively large, in the region of 0.3 mg of protein.

In 1969 Keck and Peters[37] utilised this method and reported the successful analysis of several antique and modern art specimens. Standard samples of various proteinaceous media were prepared both with and without pigment and dried at room and elevated temperature. After hydrolysis with hydrochloric acid (6 M), under vacuum, each sample was applied to a sulphonic acid resin column, which was operated at 60 °C with a pH gradient of 2.87 to 5.00 (difficult to achieve), citrate concentration gradient of 0.05 to 0.267 M and chloride cóncentration gradient from 0.18 to 0.25 M. Eluted amino acids were detected by optical density with ninhydrin. The percentage amino acid content was calculated for each of the standard media samples and these results were used for the purpose of identifying the unknown samples from works of art: this method facilitated the differentiation between gelatine, casein, glair, tempera and even horn found in the samples.

Sack *et al.*[38] analysed samples of adhesive and binding media from an ancient Egyptian painting using the ion exchange chromatographic method described by Keck and Peters[37]: amino acid compositions revealed the presence of wheat starch and gelatine.

There are, however, a number of major disadvantages associated with ion exchange chromatography in this area, namely the high cost and need for a relatively large sample. Specific single-purpose ion exchange chromatographs and columns are expensive, thus unattainable to the majority of museums and galleries, where both space and funds are at a premium so instrumentation must be of maximum versatility to be justifiable: reverse phase high performance liquid chromatography (RP-HPLC) and gas chromatography (GC) are techniques routinely used for many different analyses and are therefore more cost effective options to those on a rigid budget. In addition, ion exchange chromatography involves a lengthy and complex method of sample preparation (with additional clean up procedures), where the required sample size of paint (between 1 and 4 mg to give 300 μg of proteinaceous material) is considered large when put into the context of a fragment to be removed from a valuable work of art.

Amino acids are non-volatile and therefore cannot be directly analysed by GC, however the GC analysis of amino acids, obtained *via* the hydrolysis of proteinaceous material under acid conditions, has been achieved using trimethyl silyl derivatives.[39,40] Amino acids form two products when silylated, since the carboxyl group is easier to silylate than the amino group: under mild conditions, using hexamethyldisilazane (HMDS) as the silylating agent, the major product is the silyl ester.

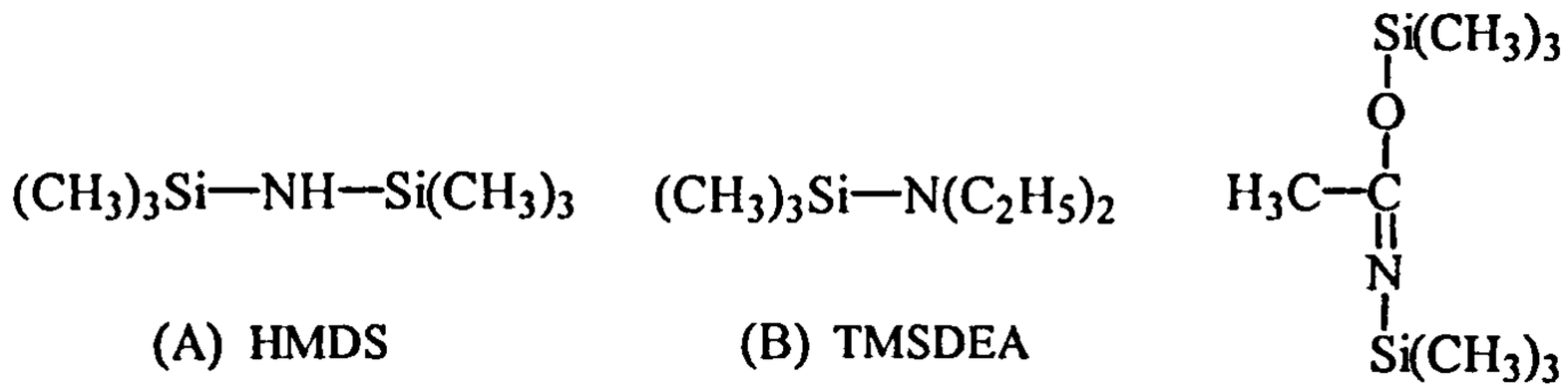


Figure 2.1: *Amino Acid Silylating Agents: (A) Hexamethyldisilazane, (B) Trimethylsilyldiethylamine & (C) N,O-bis(trimethylsilyl) acetamide*

A stronger donor is usually required by the amino group and silylation has been achieved using *N*-trimethylsilyldiethyl amine (TMSDEA) or *N*,O-bis(trimethylsilyl) acetamide (BSA), yielding the silylamine-silyl ester.

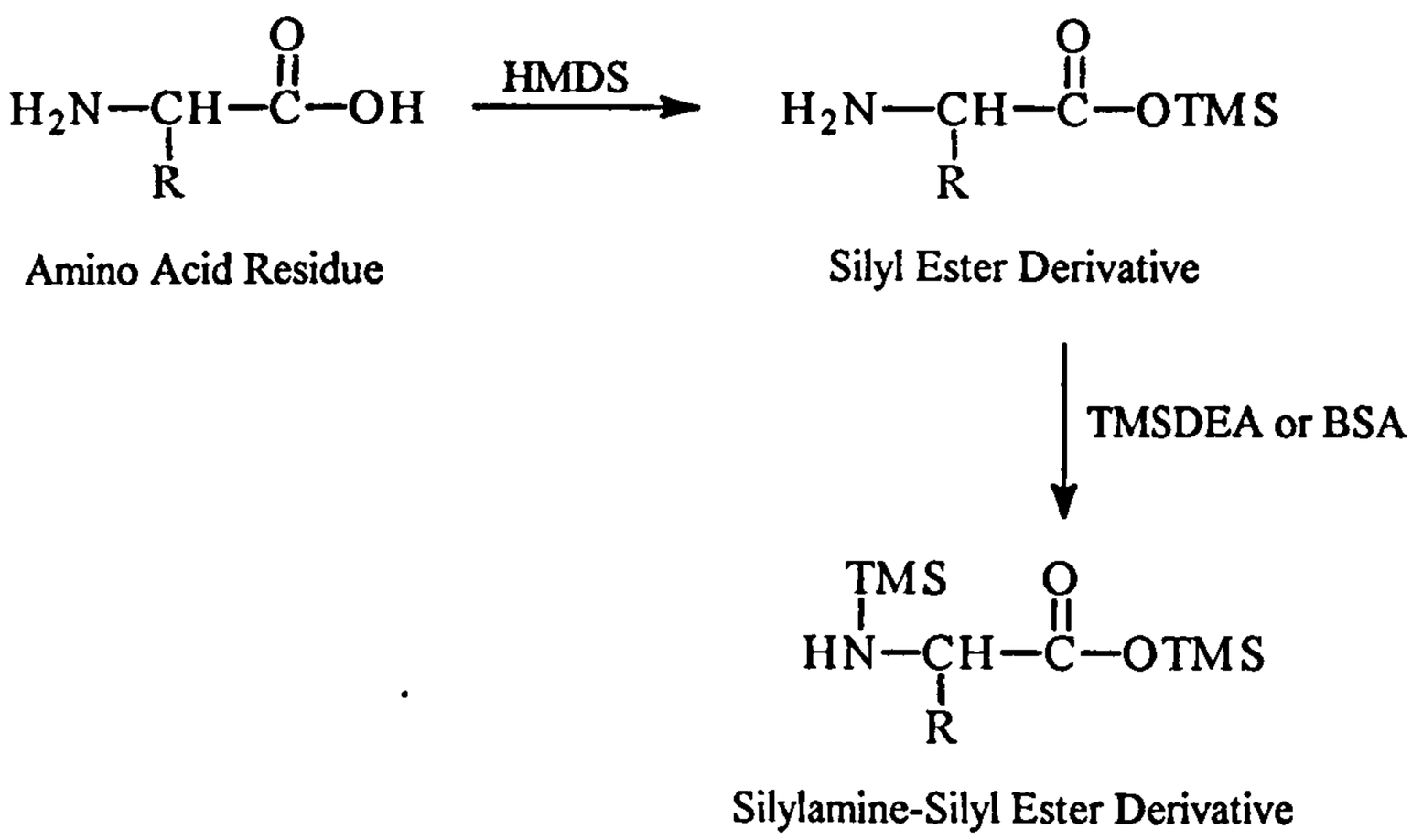


Figure 2.2: *Reaction Scheme for Amino Acid Silylation*

Masschelein-Kleiner[40] published experimental details of a GC method for the analysis of trimethyl silyl derivatives of amino acids. BSA was used to derivatise pure amino acids and those obtained from the hydrolysis of proteinaceous binding materials, namely animal gelatine, casein emulsion, egg albumin and egg yolk. The volatile derivatives were injected onto a silanised *Chromasorb W* (packed silica; high loading liquid phase) column, with a temperature programme of 90 °C to 220 °C at a rate of 2 °C per minute. The relative amounts of the constituent amino acids were determined, thus enabling the characterisation of aged proteinaceous media.

Further GC analyses of amino acids utilise their volatile *N*-trifluoroacetyl butyl ester and methyl ester derivatives.[41,42] Kenndler *et al.*[41] reported on a method for the hydrolysis of proteinaceous binding media from primings and paint layers of 16th and 18th century easel and wall paintings. Efficiencies of hydrolysis of proteins by hydrochloric acid and an ion-exchange mechanism were compared as part of the study. Derivatisation of the amino acid residues was achieved in two stages: firstly, the derivatisation of the carboxylic group, then the amino group. The reaction is illustrated in figure 2.3. The carboxylic acid functional groups were converted into butyl esters by the addition of butanol (containing dissolved hydrogen chloride, concentration 3 mol l^{-1}) and the amino groups were then acylated with trifluoroacetic anhydride, using dichloromethane as the solvent. After evaporation of the solvent the residue was dissolved in ethyl acetate, which contained an internal standard, and aliquots of the sample were injected directly onto the column (capillary *DB-5*, 5 % methyl silicone). A linear

temperature gradient programme from 100 °C to 280 °C at 10 ° per minute achieved separation of the amino acids and, by determining the relative peak areas of each, a characteristic profile of amino acids in each of the binding media was revealed.

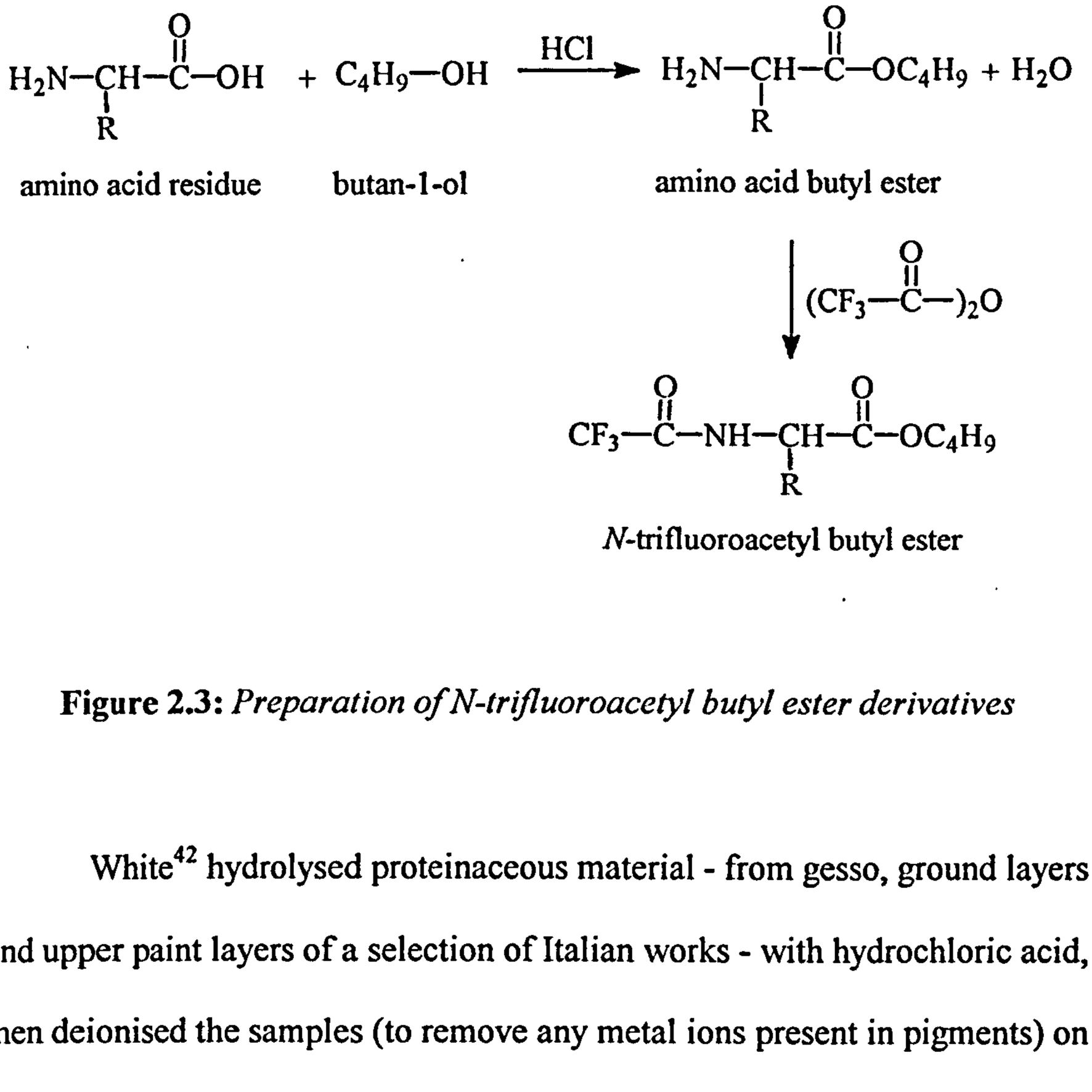


Figure 2.3: *Preparation of N-trifluoroacetyl butyl ester derivatives*

White[42] hydrolysed proteinaceous material - from gesso, ground layers and upper paint layers of a selection of Italian works - with hydrochloric acid, then deionised the samples (to remove any metal ions present in pigments) on a small column of *Amberlite IR-120H* ion-exchange resin. Methylation of the carboxylic acid functional group was achieved with methanol in the presence of anhydrous hydrogen chloride gas, then the amino group was acylated with trifluoroacetic anhydride. Samples were concentrated under nitrogen, taking care not to evaporate to dryness as *N*-acetylmethyl esters of amino acids are extremely volatile and some loss of analytes would occur. Aliquots of sample

were injected onto the column (packed, 1 % XE60 cyanosilicone gum on 100/120 mesh *Diatomite CQ* support) and a linear temperature gradient, from 80 °C to 210 °C at a rate of 3 °C per minute, achieved separation of the amino acid components. Relative peak areas were used to establish an amino acid fingerprint for each of the protein media types and characteristic amino acid ratios were used to confirm their identities.

There are, however, problems associated with these methods. The latter technique requires a sample approximately three times the size of that for analysis of oil media, since compensation should be made for losses incurred at every stage of the sample preparation. It should also be noted that losses can occur during acid hydrolysis: the presence of sugars and carbohydrates in the sample can cause the elimination of amino acids as humins, which cause darkening of the hydrolysate and the formation of insoluble matter. However, the use of an internal standard can overcome the problems associated with analyte loss in quantitative analyses.

Humins is the collective term for a mixture of coloured compounds (resembling naturally occurring melanins) produced during the acid hydrolysis of many proteins. A major contributory factor in the formation of humins is the presence of tryptophan and amino sugars (*e.g.* galactosamine, glucosamine) or carbohydrates. These compounds undergo extensive degradation during acid hydrolysis, resulting in humin production, possibly *via* the Maillard reaction.[43]

Humin formation may be prevented if hydrolysis is performed in 80 % aqueous ethanol, in the presence of an ion-exchange resin, for up to 10 hours at 95 °C.[44] It is thought that the amino acids are effectively removed from the

solution and retained on the resin by their nitrogen function, hence hindering the formation of Schiff's base condensation products between the sugars' keto/aldehyde groups and the amino groups. The immobilised amino acids can be eluted from the ion-exchange resin by simply eluting with 10 % ammonium hydroxide solution.

The fatty acid content of hens' eggs has been quantified by Mills and White[45] *via* GC analysis: the presence of tempera can be confirmed by the absence of azelate along with the presence of both saturated palmitates and stearates. Eight-year-old paint films, containing lead white and egg yolk medium, were analysed following saponification with potassium hydroxide and methylation of the acids with diazomethane. The conditions used (*i.e.* wide bore *BP-1* [methyl silicone] column with on-column injection, temperature programme from 110 °C to 310 °C at a rate of 7 °C per minute) revealed the presence of the methyl esters of saturated palmitic and stearic acids, plus a variable amount of unsaturated oleic acid. The authors report that egg fats contain only small quantities of unsaturated acids and the formation of a little azelate is not uncommon in a tempera medium, although amounts can range from negligible to almost a third of the palmitate present: in a pure oil film, the azelate peak would be at least equal to that of the palmitate methyl ester. This obviously leads to ambiguity in the results, further increased when non-drying oils are actually added to a blood-glue preparation.[46] Skans and Michelsen[47] observed that some animal glue preparations can contain up to 10 % non-drying oils, hence it is not inconceivable that a sample could be wrongly identified as egg yolk.

Nowik[48] reported on a GC method which facilitated the simultaneous analysis of both amino acids and fatty acids, which may be found together in mixed media such as tempera. Samples of proteinaceous and oil media were acid hydrolysed (6 M HCl) then neutralised with calcium carbonate. The samples were treated with an aqueous ethanol/pyridine solution prior to derivatisation which was achieved with ethyl chloroformate (ECF), then the volatile *N*-(O, S)-ethoxycarbonyl ethyl ester derivatives were extracted with chloroform (containing 1 % ECF). Samples were injected (split ratio 1:20) onto a *CP Sil-19 CB* (14 % cyanopropyl phenyl, 86 % dimethyl polysiloxane) capillary column, with a temperature programme of 100 °C to 300 °C at a rate of 30 °C per minute. This same method was previously reported for the analysis of amino acids alone: the investigations involved comparisons of methods of sample preparation, analysis and column type.[49]

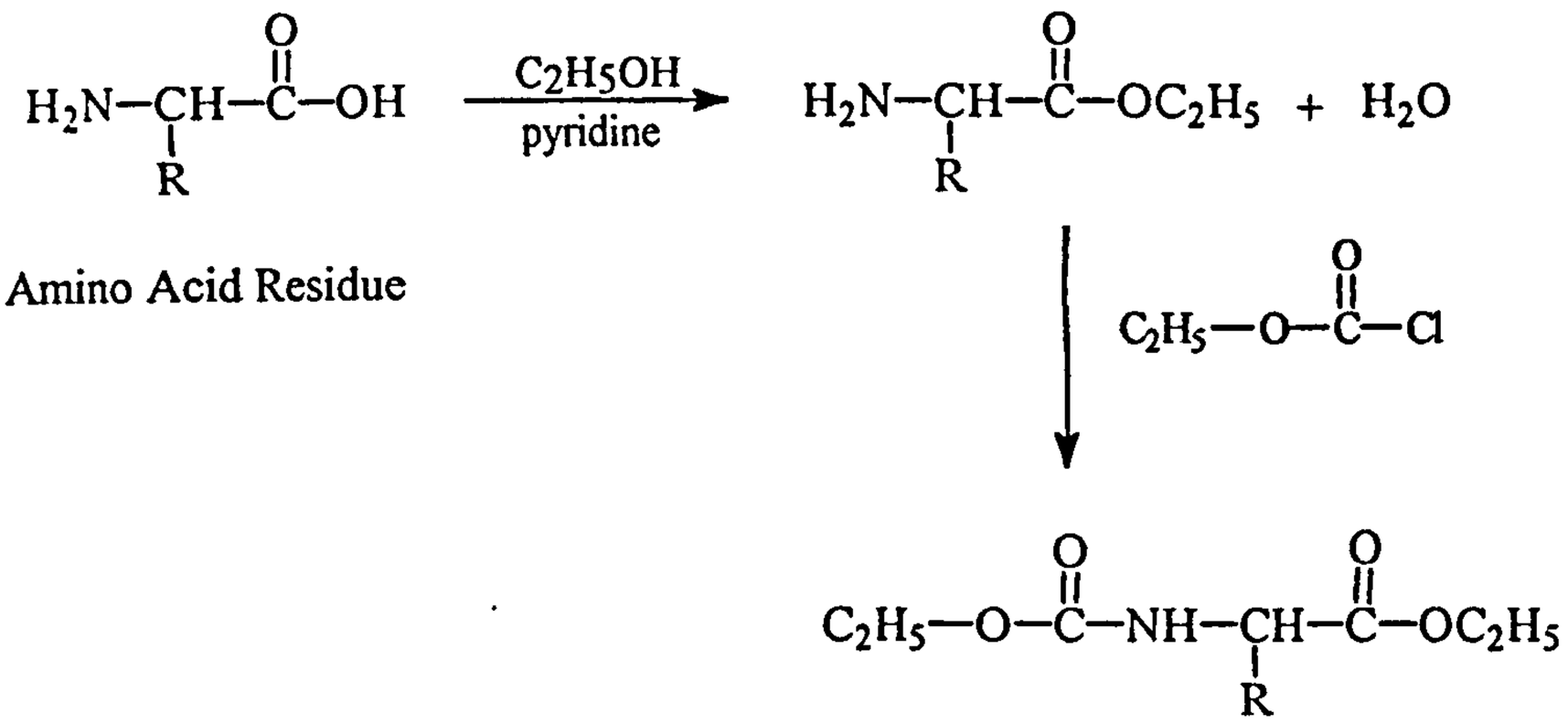


Figure 2.4: *Ethoxycarbonyl Ethyl Ester Amino Acid Derivatives*

Schilling *et al.*[50-52] recently reported on the GC analysis of ethyl chloroformate derivatives of amino acids from proteinaceous media: the studies also included investigations into the effects of pigments and ageing on the identification of proteins from art objects and details of a statistical approach to the interpretation of experimental results. Derivatisation was performed as described above, except that acid hydrolysis of the samples was achieved using constant boiling hydrochloric acid (6 M) under vacuum conditions. Samples were injected onto an *HP-INNOWAX* (bonded polyethylene glycol wax) capillary column with a temperature programme of 70 °C to 250 °C at a rate of 27 °C per minute.

In recent years the analysis of amino acids by RP-HPLC has developed significantly, primarily due to the speed and sensitivity of the technique compared with more traditional specialised amino acid analysers. Currently, RP-HPLC following pre-column derivatisation is one of the most widely used methods of analysis of amino acids, since it provides a good selection of both derivatising agents and detection techniques. The technique lends itself well to the field of conservation science: the occurrence of proteinaceous material in art objects coupled with the prohibitively small samples characteristic of the work, means that RP-HPLC is an ideal tool for the identification of samples taken from works of art.

Erhardt *et al.*[53] reported a method for the analysis of phenylthiocarbamyl derivatives of amino acids from proteinaceous material taken from art objects. Samples were hydrolysed under acid conditions, dried, neutralised with triethylamine, redried then derivatised with phenylisothiocyanate (PITC). The derivatisation reaction was stopped by the

evaporation of the PITC and the samples were analysed by RP-HPLC after dissolution in aqueous phosphate buffer. Samples were injected onto a C18 column and a ternary solvent system was used as the mobile phase (water/acetonitrile/acetate buffer), with a gradient programme from 0:6:94 to 16:28:56. A reasonable separation of the amino acids is shown in the paper, but there is very little discussion about the results themselves.

Halpine[54,55] has made extensive use of RP-HPLC for the amino acid analysis of proteinaceous matter from objects of artistic importance. In a recent study,[54] samples taken from *The Annunciation with St. Francis and St. Louis of Toulouse*, a series of 15th century Italian tempera panels by Cosimo Tura, were submitted for amino acid analysis using the *Picotag*[56] system, which was selected on account of its flexibility and good reproducibility with very small samples. Hydrolysis of the protein material was performed with acid vapour and derivatisation of the liberated amino acids, following buffering and drying of the hydrolysate, was achieved with phenylisothiocyanate (PITC). The samples were analysed by RP-HPLC, utilising *Picotag*[56] chemistry, with a binary solvent system of acetate buffer and acetonitrile. A C18 column was used with a gradient programme to separate the 18 amino acids deemed necessary for the identification of proteinaceous material. The use of nor-leucine, an unnatural amino acid, as an internal standard facilitated the quantification of the constituent amino acids and, using this method, a number of samples of animal glue media and egg/glue media were identified. A problem with this method is in the choice of derivatising agent: the PITC-amino acid derivatives degrade in solution

and so must be kept at -4 °C until required for analysis. Only then can the samples be solubilised in the dilution buffer.

Concern has been expressed with respect to the effects of mineral pigments on the reproducibility of the analyses.[57] Halpine[58] performed tests in which copper- and calcium-based pigments were added separately to proteinaceous reference samples, confirming that the presence of copper lowers the yield of all the amino acids whilst calcium reduced the recovery of aspartic and glutamic acids. This means that calcium could hinder the identification of a casein medium and copper could lower the amino acid levels to those associated with background interference, making interpretation of analytical results more difficult. It has been proposed that the copper somehow affects the hydrolysis procedure, but an alternative explanation is that it in fact prevents complete derivatisation of the amino acids in the hydrolysate due to the formation of copper-amino acid complexes (see chapter 6, section 6.1.4).[59]

It is desirable to remove any pigment from the sample, if possible, and Halpine achieved this using an extraction method.[55] An egg tempera panel with gesso ground, which had been prepared in the laboratory, was sampled and 30 µl of HPLC grade water was added to each sample in a hydrolysis tube. After thorough mixing the samples were left to stand for 1 hour, sonicated to break any remaining large particles, then centrifuged for a maximum of 15 minutes at high speed. The water soluble proteinaceous material was then removed, evaporated to dryness and analysed by RP-HPLC after derivatisation with PITC, in addition to the insoluble material which

remained in the precipitate. This simple technique, besides removing unwanted contaminants from the samples, proved useful for the determination of mixed media, since the degree of water solubility was found to vary depending on the protein types present.

It must be remembered that paintings are not the only art objects of importance for analysis - stone sculptures, icons, frescoes, stuccoes and wall-paintings provide other sources of samples posing interesting questions for the conservator. In particular, protein levels in these samples are typically much lower than those seen for easel paintings, for example, so the sensitivity of the method of analysis is paramount. Ronca[60] used RP-HPLC, following derivatisation of the amino acid residues with PITC, to analyse both artificial samples and those taken from a number of 13th century French stone sculptures and Italian stuccoes, a 16th century Italian external fresco and the gesso ground of a 15th century Italian wooden statue. Proteinaceous material was extracted from the matrices by a variety of chemical methods, the most successful being the use of 1 N sodium hydroxide solution at 80 °C for 3 hours followed by colourimetric determination with Folin's reagent. The amino acid composition of the extracted protein was achieved *via* RP-HPLC, preceded by direct acid hydrolysis of the proteinaceous material, desalting of the hydrolysate using a sulphonic acid resin column and final derivatisation with PITC. These rather protracted analyses revealed the presence of gelatine and egg proteins in the samples, which corroborated historical information on the nature of the materials commonly used by artists in those periods.

The use of 9-fluorenylmethyl chloroformate (FMOC) as a derivatising agent in the RP-HPLC analysis of proteinaceous media has been reported by

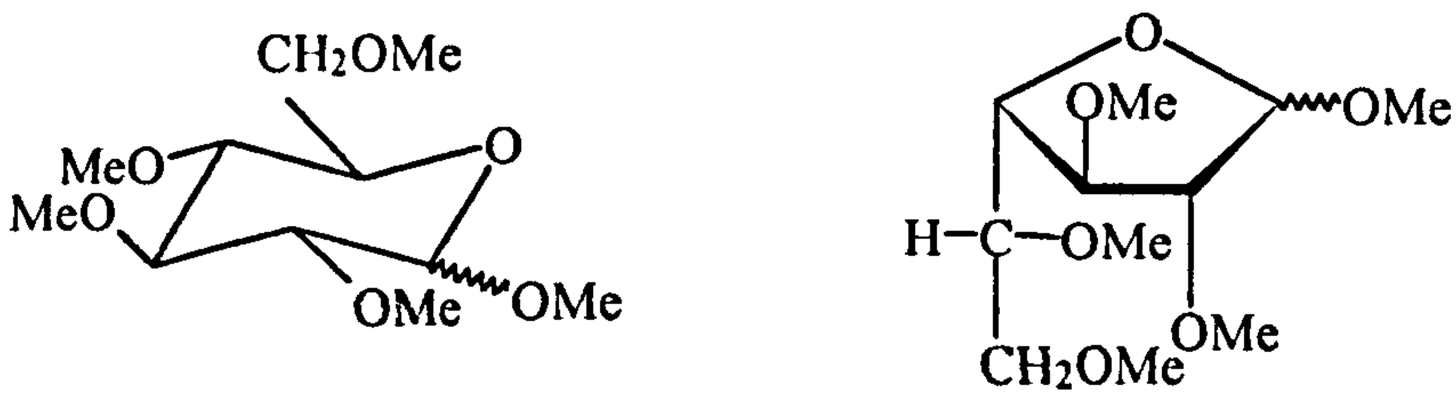
Grzywacz.[61] Samples were hydrolysed under acid vapour conditions, dried and diluted with borate buffer (pH 8.5) prior to derivatisation with FMOC. Samples were injected onto a *Spherex* 3 μm C18 (ODS) column with a binary gradient elution programme based on the method developed by Haynes[62]: the eluents were (A) 50 mM sodium acetate and 7 mM triethylammonium acetate with 10 % acetonitrile, adjusted to pH 6.5 with acetic acid, and (B) acetonitrile/water (90:10, v/v). Standard proteinaceous media and a number of museum samples were analysed and identified using this method.

2.4 Analysis of Gum Media

Chemists have employed a variety of analytical techniques for the characterisation of carbohydrate compounds, probably the most widespread over recent years being gas chromatography (GC). The first reported use of GC for these analyses was in 1958, when McInnes[63] published work on the separation of the fully methylated methyl glycosides of a selection of monosaccharides. Since GC is dependent on the volatility of the analytes, it is necessary for the sugars to undergo some form of derivatisation reaction prior to analysis.

Bishop[64] investigated the properties of a number of derivatives, including fully and partially methylated methyl glycosides, acetates, acetals and trimethylsilyl ethers. The permethylated methyl glycosides used had long retention times, so greater volatility of the derivatives was required. This was achieved by reducing the sugars with sodium borohydride prior to acetylation, thus forming the more volatile alditol acetate derivatives.[65] This derivatisation method has the advantage of producing single peaks for each

sugar treated,[66] facilitating the separation of relatively complex mixtures, but the actual procedure is lengthy and complicated.



Me = CH_3

Figure 2.5: *Examples of Permethylated Methyl Glycoside Derivatives*

"Methylation analysis" of polysaccharides was made possible *via* the GC separation of a number of methylated alditols as their corresponding acetate derivatives.[67] Investigations into the position of glycoside linkages and the composition of sugars became possible, following methylation of polysaccharide free hydroxyl groups, hydrolysis and the subsequent analysis of the partially methylated monosaccharides thus formed.[68]

Trimethylsilyl (TMS) derivatives of carbohydrates are amongst the most widely used, since their preparation is relatively simple and they are even more volatile than those discussed above. The publication, by Sweeley *et al.*,[69] of investigations into the GC analysis of trimethylsilyl sugar derivatives, from mono- to tetra-saccharides, sealed the popularity of this procedure and oligosaccharides as large as octaose have been successfully analysed in the form of their TMS derivatives.[70]

In 1979 Honda *et al.*[71] reported on a method of trimethylsilylation which used hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) as the silylating agents: TMCS was used as a catalyst to enhance the silylating power of HMDS. Derivatisation was complete within an hour, but the initial hydrolysis of the sample was convoluted, requiring heating for 5 hours under nitrogen before evaporating to dryness. The gums, arabic and tragacanth, were analysed using this method, but the lengthy sample preparation made it less than ideal.

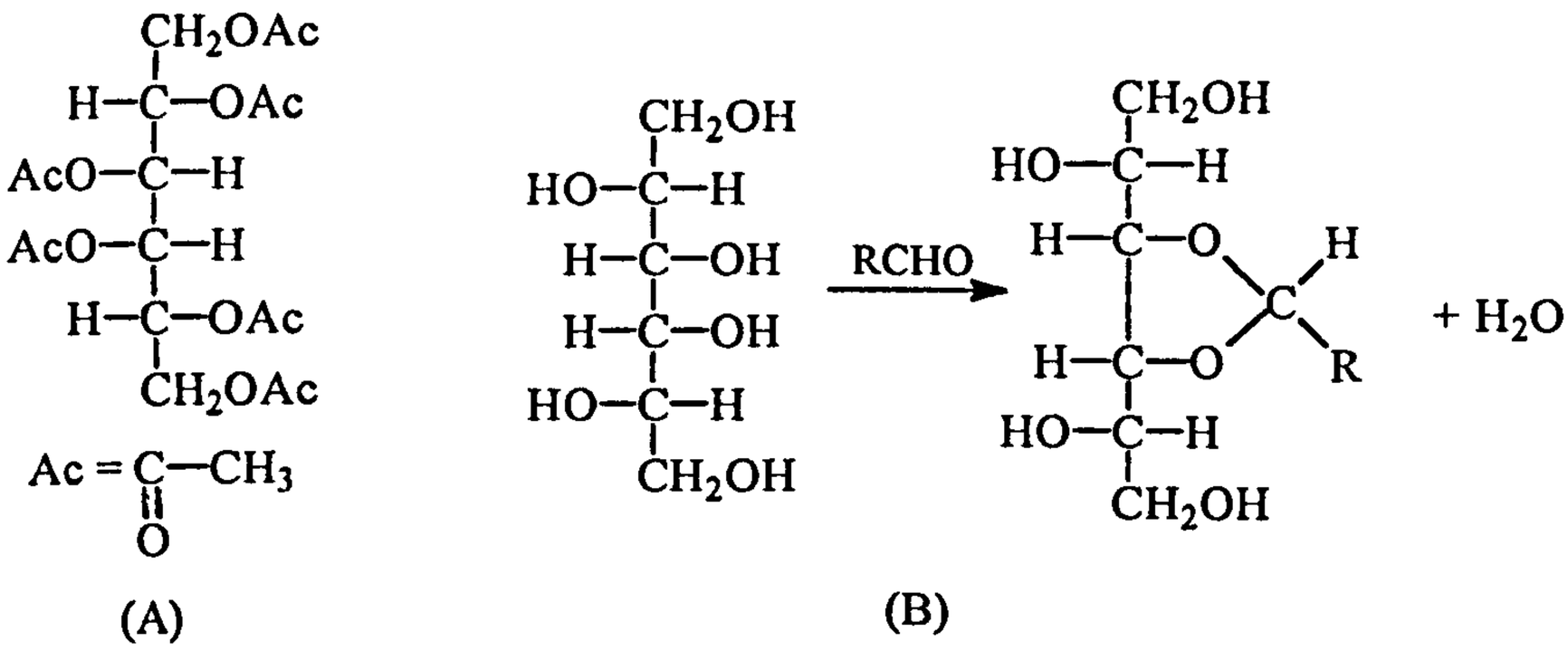


Figure 2.6: *(A) Alditol Acetate Derivative &*

(B) Acetal Derivative Formation

A pyridine solution of *N*-trimethylsilylimidazole was used by Twilley[72] in 1984. Hydrolysis was performed at 100 °C for 18 hours, then the samples were passed through an ion exchange column. Any water present in the samples was removed by freeze drying over phosphoric anhydride under vacuum. However, when the method was used for the analysis of relatively

large botanical samples, *i.e.* 50 mg, the chromatograms did not show any of the uronic acid components.

In 1977 Sullivan and Schewe[73] reported on the use of trifluoroacetylated derivatives for the analysis of sugar syrups. *N*-Methylbis(trifluoroacetamide) (MBTFA) in pyridine was used as the derivatising reagent, the sample being heated, with occasional shaking, for 60 minutes. The derivatives were separated on a polar OV-210 column (trifluoropropylmethyl silicone stationary phase).

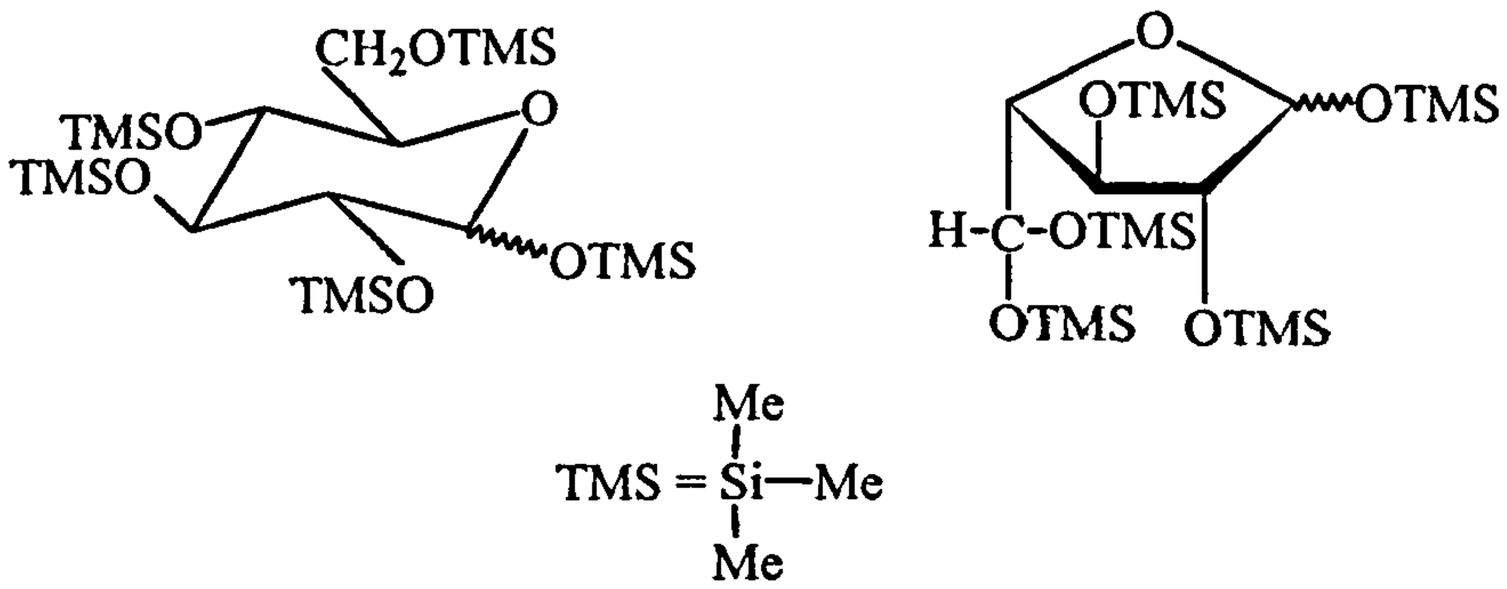
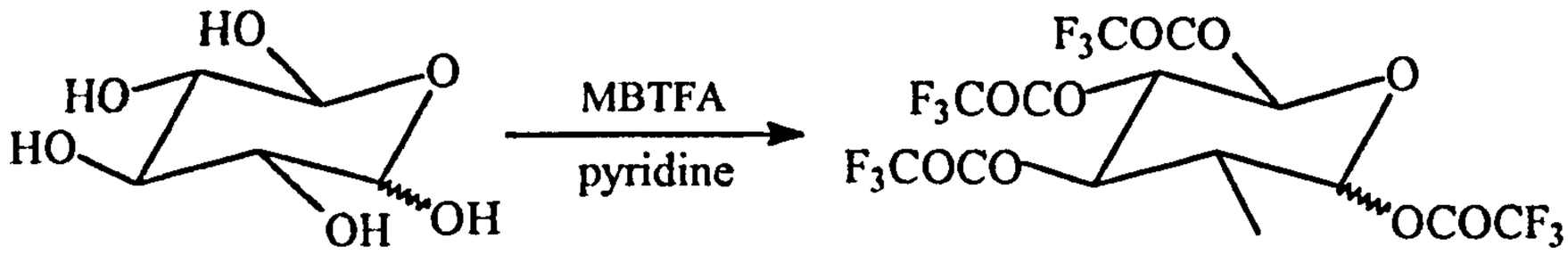


Figure 2.7: *Trimethylsilyl Glycoside Derivatives*

Trifluoroacetylated oximes were used by Decker and Schweer[74] in 1982: the derivatives were prepared rapidly at low temperature and separation by GC was achieved with relatively low column/oven temperatures.

Aldononitrile acetate derivatives are also prepared quickly, *via* synthesis of the oxime intermediate and simultaneous dehydration to the nitrile form. Final acetylation with acetic anhydride is performed at 60 °C, the whole derivatisation process taking around 15 minutes to complete.[75]

Importantly, the presence of water in the reaction mixture does not affect the derivatisation process[76] and the aldononitrile acetate derivatives are more stable than the corresponding TMS aldononitriles, storage for several months at room temperature in a vacuum desiccator being practicable.[77]



$MBTFA = (CF_3CO)_2N{-}CH_3$

Figure 2.8: *Trifluoroacetylated Glycoside Derivative*

Sugars and uronic acids were analysed simultaneously as trimethylsilyl methyl glycosides by Ha and Thomas.[78] Methanolysis of the uronic acids was performed, then the neutral sugars were derivatised with a mixed TMS reagent (HMDS and TCMS in pyridine): the method proved to be sensitive for small samples, but the lengthy methanolysis (80 °C for 16 hours) meant that it was less than convenient. In addition, as with many TMS reactions, the presence of water was found to lead to incomplete silylation. A similar method, employing methyl glycosides, was investigated by Aspinall *et al.*[79,80] in their study of *Auracaria bidwilli* gum, but again the methylation procedure was extremely protracted.

Reinhold *et al.*[81] synthesised per-*O*-trimethylsilyl carbohydrate boronates and analysed the derivatives by gas chromatography coupled with

mass spectrometry (GC-MS). Stereospecific carbohydrate derivatives were prepared using pyridine solutions of either alkane- or arene-boronic acids, prior to silylation with trifluorobis(trimethylsilyl) acetamide and chlorotrimethylsilane mixed in equal quantities. All the derivatives, except the methyl D-galactosides, gave a single peak: this factor, when considered with the relatively low temperatures required for separation, means that the method is useful for the quantitative characterisation of carbohydrates.

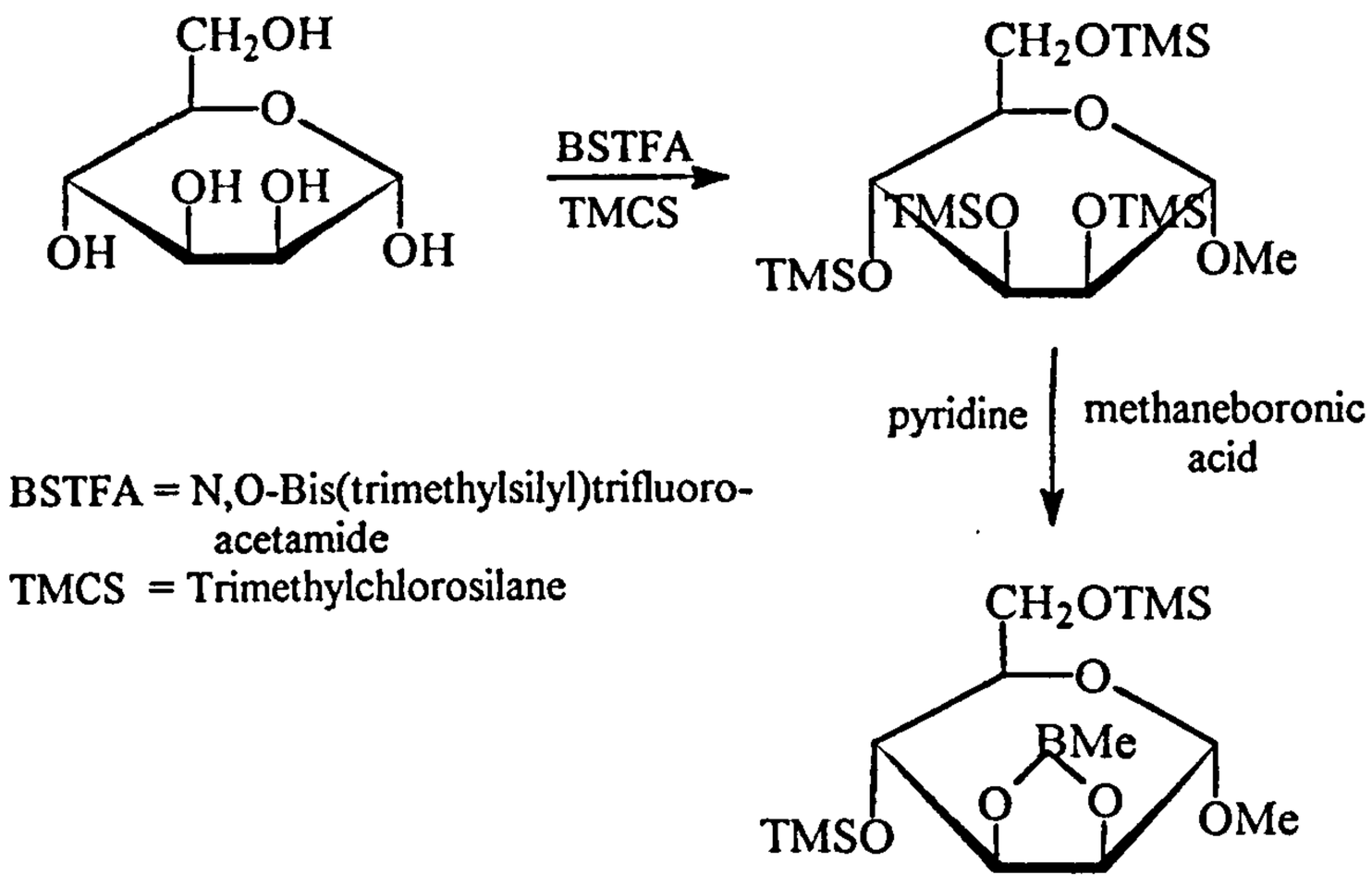


Figure 2.9: *Example of Derivatisation Reaction Scheme*

Wiecko and Sherman[82] used the monoacetylhexose cyclic boronic esters of carbohydrates for their study of GC-MS behaviour, characterising the derivatives of sugars including D-glucose, D-mannose and D-galactose by this method.

An obvious problem associated with the use of any of these methods for the analysis of gums used in works of art is the sample size: many of these

techniques will not be sufficiently sensitive for the sub-milligram samples which are available to the conservation scientist. Despite this, there has been some progress in the analysis of gum media from art objects using these instrumental techniques, often in conjunction with thin layer chromatography (TLC).

Masschelein-Kleiner *et al.*[83,84] used TLC and GC to analyse the trimethylsilyl derivatives of sugars resulting from the hydrolysis of samples of the surface coating of a wooden Egyptian sarcophagus, dating from the 21st Dynasty. A mixed alumina-silica stationary phase was prepared for TLC, the eluent being a mixture of propanol, ethyl acetate, water and 25 % ammonia solution: naphthoresorcinol was used for detection on the plates after separation. Separation and analysis by GC were achieved using an E301 silicone column (length 2.0 m, external diameter 3 mm) with a temperature programme from 160 to 200 °C at 1.7 °C min^{-1}. The analysis disclosed the presence of gum tragacanth and, when the paint medium itself was analysed, a mixture of honey and gum tragacanth was revealed.

Flieder[85] utilised TLC to separate and identify gum media from a 16th century manuscript. The gum samples were hydrolysed under acidic conditions, deacidified with an ion-exchange resin, then separated on a silica plate using butanol-ethanol-water (57:27:16) as the eluent; the sugar components were visualised with naphthoresorcinol. Although not every component was separated, the identification of the medium as gum arabic was facilitated.

Birstein[86] used a combination of TLC and GC plus infra-red (IR) spectroscopy when studying problems associated with the binding media

found in Asian wall paintings. Hydrolysates of samples (1-5 mg of polysaccharide material) were acetylated prior to analysis but, although the technique was sensitive enough to facilitate identification of the media studied, the sample preparation was lengthy, requiring 12 hours for hydrolysis and 5 hours for derivatisation. Birstein and Tul'chinsky[87] employed IR techniques for polysaccharide identification in archaeological samples; spectra of artistically important gums and some samples removed from works of art were published, but the results were not particularly informative, only allowing the distinction between polysaccharides and water-soluble proteins. Furthermore, large samples were required in order to obtain any spectra.

Szyszko[88] investigated the nature of binding media used in the paint layers of three Egyptian epitaphal stelae, on wooden supports, two of which dated from the second millenium BC and the third thought to be from a much later period. TLC was used in these investigations, the results of which indicated gum tragacanth as the binding medium.

Studies of paintings found in the Tomb of Nefertari[89,90] at Luxor revealed an interesting local phenomenon: the water-soluble paint medium used in the works was found to contain no rhamnose component, which is usually indicative of gum tragacanth. However, when samples of gum taken from locally growing trees of the *Acacia* genus were analysed by GC they too were found to be lacking in rhamnose, whilst the remainder of the sugar content matched that seen in the samples taken from the paintings. It was therefore concluded that the paint medium was in fact gum arabic, despite all other commercial sources of the gum possessing a rhamnose component. These findings could in turn mean that the samples from the Egyptian

epitaphal stelae could actually contain gum arabic from the same local source, rather than gum tragacanth as originally concluded.[91]

Twilley[72] published a report on the analysis and artistic applications of plant gums. Analysis of samples was achieved *via* a number of techniques, including TLC and GC analyses of trimethylsilyl sugar derivatives.

Erhardt *et al.*[53] presented their findings of investigations into the GC analysis of gums to the American Institute for Conservation of Historic and Artistic Works (AIC). Samples were hydrolysed with trifluoroacetic acid (2.3 ml in 7.7 ml water), then dried in a vacuum desiccator. The sugars were then converted to their oxime form with a pyridine solution of hydroxylamine hydrochloride prior to silylation with trimethylsilylimidazole. Samples were separated on a non-polar DB1 (bonded polydimethylsiloxane) capillary column with a temperature gradient programme from 100 °C to 275 °C at 10 °C per minute. Resolution was improved using a more polar DB17 column, although higher temperatures were required - 150 °C to 325 °C at 10 °C per minute.

In 1996 Bleton, Coupry and Sansoulet reported on a GC method for the analysis of ink samples from ancient manuscripts.[92] Following methanolysis and silylation with trimethylsilylimidazole, samples were injected onto a SE52 (5 % diphenyl, 95 % dimethyl polysiloxane) capillary column with a stepped temperature programme of 40 to 130 °C at 9 °C per minute, then 130 to 290 °C at 2 °C per minute, remaining at 290 °C for a further 30 minutes. Reference samples of old ink and samples taken from a variety of manuscripts were analysed thus.

Other chromatographic techniques can be utilised for the characterisation of sugar based compounds. Pyrolysis-mass spectrometry (Py-MS) was the technique employed by Wright[93] in studies of Egyptian mummy cases. Samples of organic materials used in the construction of the cartonnages were examined by Py-MS: pyrograms were compared with those of a series of standard materials, facilitating the identification of around 50 % of the samples. Polysaccharide gums were detected in samples from objects between 2000 and 4000 years old.

Derrick and Stulik[94] used pyrolysis-gas chromatography (Py-GC) to investigate the separation and identification of natural gums in works of art. Powdered gum samples were pyrolysed using a coil type probe: standard gum samples were pyrolysed at 700 °C, but this temperature was lowered for the analysis of more complex samples. Gums arabic, tragacanth, guar, ghatti and karaya all gave distinguishable and reproducible pyrograms, allowing their identification. It was observed that the pyrograms of gum-pigment mixtures differed from those of the standard gums, their peak patterns and intensities being altered. This effect was minimised by performing the pyrolysis of the samples at the lower temperature of 400 °C. Computational methods of pattern recognition were employed to assist with sample identification.

In 1985 Williams and Langdon[95] used gel permeation chromatography (GPC) to characterise gum arabic, identifying the three principal mass components of the gum.

Ion exchange chromatography, be it anion or cation, was popular for a time, but proved to be less sensitive than other HPLC methods.[96-98] In 1976

Rabel[99] reported on a normal phase partition HPLC method, using refractive index (RI) detection, for sugar determination which was sensitive to levels around 80 μg: however, much of the work on the use of HPLC techniques for the analysis of sugars was recorded between 1980 and 1987.[100-107] A variety of derivatisation techniques (*e.g.* postcolumn cuprammonium, dansylhydrazine, precolumn dabsylhydrazine) and detection methods (*e.g.* UV absorption, fluorescence, RI) have been used and sensitivities as low as 5 pmol have been achieved. It must be noted, however, that none of these latter studies were in the particular area of conservation science.

Analytical methods used for the determination of polysaccharides and proteins in art objects have been reviewed previously,[108,109] the most recent paper focusing on chromatographic techniques.[110]

CHAPTER 3

Experimental

3.1 Analysis of Proteinaceous Media by RP-HPLC

3.1.1 Samples

Specimens of standard proteinaceous materials were studied in order to obtain a library of standard chromatograms for comparison with "real" samples removed from works of art. The materials used were casein, hens' eggs albumin, hens' eggs yolk, whole hens' eggs, sturgeons' glue, parchment for glue making, gelatine sheet, bone pearl glue, rabbit skin glue and high strength skin glue (A.P. Fitzpatrick, except egg yolk/whole egg). Aqueous solutions of the samples were painted onto microscope slides and allowed to dry naturally, at room temperature and in daylight, for a week: fresh samples of each material were also used.

The Tate Gallery, London, supplied a selection of both thermally aged and unaged samples of proteinaceous media, mixed with umber, painted over C19 oil priming. In addition, a set of artificially aged egg tempera paint samples were prepared by the Courtauld Institute's Department of Conservation and Technology for an investigation into the effects of ageing and pigment interferences. The pigments (lead white, vermilion, azurite and verdigris) were ground with water and subsequently egg yolk medium, then moderately thick, uniform layers were painted on to Melinex (polyester) film. After drying, the films were subjected to varying degrees of accelerated ageing *via* prolonged exposure to heat and light, whilst the pure egg yolk medium control samples were stored in the dark at room temperature. Two

levels of light ageing were achieved using a Xenon-arc Fade-o-meter: one set of samples was left for 413 hours with no thermal ageing whilst a second set was exposed for 192 hours, then subjected to thermal ageing at 70 °C (relative humidity 55 %) for 21 days.

Further samples, taken from various works of art, were supplied by the Tate Gallery, London and final year Conservation of Fine Art (MA) students at the University of Northumbria. Minute samples of paint media and adhesives were removed from the surface of works, with the aid of a surgical scalpel, then immediately placed in *Reacti-vials* which had been chemically cleaned for this purpose.

3.1.2 Chemical Cleaning Process

Reacti-vials (Pierce) were pre-soaked, then sonicated in a 5 % aqueous solution of Decon® (an amphoteric laboratory glassware detergent) for 30 minutes. The vials were rinsed 3 times in distilled water, sonicated for 30 minutes in distilled water, to which a small amount of concentrated hydrochloric acid (analytical grade) had been added, then rinsed 3 times with distilled water. The clean vials were finally oven dried ready for use.

3.1.3 Sample Preparation

3.1.3.1 Acid Hydrolysis

Hydrolysis of the proteinaceous material, to liberate free amino acids for derivatisation, was achieved simply by heating the sample (0.1 mm^2 approximately) with concentrated hydrochloric acid (0.1 ml, analytical grade) for 72 hours at 80 °C in a *Reacti-Therm* heating module (Pierce). A borate

buffer was prepared from a solution of orthoboric acid (1.0 M, analytical grade), the pH of which was adjusted to 6.2 with sodium hydroxide solution (30 %, analytical grade), then diluted to five times its original volume with deionised water, resulting in a pH 7.7 buffer solution. After cooling, the hydrolysate was diluted with the borate buffer (1.0 ml, pH 7.7) and the resulting solution's pH was adjusted (pH 7-8 by indicator paper) using sodium hydroxide solution (30 %, analytical grade) as required. An aliquot (0.2 ml) of aqueous ethylenediaminetetraacetic acid (EDTA) solution (disodium salt, dihydrate; 0.05 M) was added to the samples containing the copper based pigments, azurite and verdigris.

3.1.3.2 Derivatisation

A solution of 9-fluorenylmethyl chloroformate [FMOC] (0.015 M in acetone) was prepared. An aliquot (0.5 ml) of the FMOC solution was then added to an equal amount (0.5 ml) of the hydrolysed sample solution and the reaction was allowed to proceed for 30-40 seconds. The reaction mixture was then extracted with hexane (3 x 2 ml) and the combined aqueous layers containing the derivatised amino acids were refrigerated until analysis.

3.1.4 Analysis

3.1.4.1 Instrumentation

The pump was a quaternary gradient *SpectraSystem P4000* (Thermal Syndicate Inc.) coupled with a *SpectraSystem UV2000* detector and a *SpectraSystem SCM400* solvent degassing unit. The detector was operated at a wavelength of 260 nm. The manual Rheodyne injection port (model 7125)

was fitted with a 20 µl loop and the *Spherisorb ODS2* (C18) column (Phase Separations, 25 cm length) was maintained at an operating temperature of 35 °C in a temperature controlled heater-block column oven (Jones Chromatography). The acetonitrile used was gradient HPLC grade and ultra-pure water (18 MΩ) was obtained using a *Milli-Q* water purification unit (Millipore). The *Baseline 810* (Millipore) chromatography software package was utilised for data processing.

3.1.4.2 Methodology

Separation and analysis were achieved using RP-HPLC. Samples (20 µl) were injected on to the column with a mobile phase of acetate buffer and acetonitrile (MeCN). The acetate buffer was prepared by adding acetic acid (3.0 ml, analytical grade) and triethylamine (1.0 ml, analytical grade) to ultra-pure water (800 ml): the pH of the solution was adjusted to 4.2 with sodium hydroxide solution (30 %, analytical grade) as necessary, then the buffer was transferred to a volumetric flask (1 litre) and diluted to volume with ultra-pure water. A stepped gradient programme (flow rate 1 ml min^{-1}) ensured that 15 out of the 16 amino acids in the standard mixture were fully resolved. The mobile phase composition is tabulated below:

Time / minute	% MeCN	% Buffer
0	20	80
30	30	70
45	50	50
55	55	45
60	100	0

A 10 minute interval at 100 % MeCN at 1ml min^{-1}, plus equilibration for 15 minutes prior to each programme run prevented baseline interference in subsequent analyses.

3.2 Analysis of Gum Media by Gas Chromatography

A variety of methods of sample preparation and analysis were utilised for the investigation of gum media. Hydrolysis conditions, derivatising agents and instrumentation were all varied in the course of these investigations.

3.2.1 Samples

Specimens of a series of standard monosaccharides and gum materials were studied to facilitate the compilation of a library of standard chromatograms for comparison with "real" samples removed from works of art. The monosaccharides used (mannose, arabinose, rhamnose, fucose, xylose, galactose, glucose, glucuronic acid and galacturonic acid, all Merck) were in their chromatographically pure forms and the gum materials used (arabic, tragacanth, cherry, guar, ghatti, locust bean and karaya, all A.P. Fitzpatrick) were all in solid form, except arabic which was obtained in both solid and liquid forms (liquid gum from Windsor and Newton).

Aqueous solutions (10 % approximately) of the gums were prepared, painted onto glass microscope slides and allowed to dry naturally. One set of gum samples was then subjected to artificial ageing (thermal and light, 8 weeks) whilst the other set was used unaged for comparative purposes.

Samples from works of art were supplied by the Tate Gallery, London. Minute samples of paint were removed from the surface of works, taking care to include as little paper fibre as possible, with the aid of a surgical scalpel, then immediately placed in chemically clean *Reacti-vials* (see section *3.1.2*) which were sealed with caps containing PTFE coated septa.

3.2.2 Sample Preparation

3.2.2.1 Method 1

3.2.2.1.1 Hydrolysis

Hydrolysis of the standard gum materials, in order to obtain the composite monosaccharides, was achieved by heating the samples (1-2 mg weighed into *React-vials*) in a *Reacti-Therm* heating module (Pierce) for between 1 and 4 hours at 105 °C with trifluoroacetic acid (0.5 ml, AristaR®, 2.3 ml in 7.7 ml water). The sample was allowed to cool, then uncapped and placed in a vacuum desiccator, containing sodium hydroxide pellets, until dry.

3.2.2.1.2 Oxime Formation

STOX® reagent (0.1 ml, freshly prepared) was added to the *Reacti-vial* and the sample was heated at 80 °C for a further 90 minutes in the *Reacti-Therm* heating module. STOX reagent was prepared from 25 mg ml^{-1} hydroxylamine hydrochloride in pyridine, with 6 mg ml^{-1} phenyl-β-D-glucopyranoside as an optional internal standard.

3.2.2.1.3 Silylation

Trimethylsilylimidazole [TSIM] (0.1 ml, derivatisation grade) was added to the cooled sample (room temperature), which was then periodically shaken over the next 30 minutes, forming the volatile trimethylsilyl oxime derivatives of the composite monosaccharides.

3.2.2.2 Method 2

3.2.2.2.1 Hydrolysis

Each standard gum sample (1-2 mg in *Reacti-vials*) was heated for 2 hours, in a *Reacti-Therm* heating module (Pierce), at 105 °C with dilute hydrochloric acid (0.1 ml, 2 M). The sample was allowed to cool, then uncapped and placed in a vacuum desiccator, containing sodium hydroxide pellets, until dry.

3.2.2.2.2 Oxime Formation

STOX reagent (0.1 ml, freshly prepared) was added to the *Reacti-vial* containing the hydrolysis product and the sample was then heated at 80 °C for 90 minutes in a *Reacti-Therm* heating module (Pierce).

3.2.2.2.3 Silylation

Trimethylsilylimidazole [TSIM] (0.1 ml, derivatisation grade) was added to the cooled sample (room temperature), which was then periodically shaken over the next 30 minutes, forming volatile trimethylsilyl oxime derivatives.

3.2.2.3 Method 3

3.2.2.3.1 Hydrolysis

Standard gum media (1-2 mg in *Reacti-vials*) were heated with trifluoroacetic acid (0.1 ml, analytical grade) at 105 °C for 30 minutes in a *Reacti-Therm* heating module: the samples were then allowed to cool to room temperature.

3.2.2.3.2 Oxime Formation

STOX reagent (0.1 ml, freshly prepared) was added to the cooled samples, which were then heated for 90 minutes at 80 °C in a *Reacti-Therm* heating module (Pierce).

3.2.2.3.3 Silylation

Hexamethyldisilazane [HMDS] (0.3 ml, derivatisation grade) and trifluoroacetic acid (5 drops, analytical grade) were added to the samples, which were vigorously shaken for 30 seconds then allowed to stand for 30 minutes before analysis.

3.2.2.4 Method 4

3.2.2.4.1 Silylation

Pyridine (0.2 ml, analytical grade) was added to samples of individual, chromatographically pure monosaccharides (<1 mg), then HMDS (0.3 ml, derivatisation grade) and trifluoracetic acid (5 drops, analytical grade) were

added. The samples were vigorously shaken for 30 seconds then allowed to stand for 1 hour prior to analysis.

3.2.2.5 Method 5

3.2.2.5.1 Hydrolysis

Standard gums (1-2 mg in *Reacti-vials*) and individual monosaccharide samples (<1 mg in *Reacti-vials*) were heated in a *Reacti-Therm* heating module (Pierce) at 105 °C, for 30 minutes and 10 minutes respectively, with trifluoroacetic acid (0.1 ml, analytical grade). The samples were allowed to cool to room temperature before pyridine (0.2 ml, analytical grade) was added to the *Reacti-vials*.

3.2.2.5.2 Silylation

Hexamethyldisilazane [HMDS] (0.3 ml, derivatisation grade) and trifluoroacetic acid (5 drops, analytical grade) were added to the samples, which were vigorously shaken for 30 seconds then allowed to stand for 1 hour prior to analysis.

3.2.2.6 Method 6

3.2.2.6.1 Hydrolysis

Samples removed from works of art (0.1 mm^2 approximately) were placed directly into *React-vials* then heated for 30 minutes at 105 °C with trifluoroacetic acid (0.1 ml, analytical grade) in a *Reacti-Therm* heating

module (Pierce). The samples were allowed to cool to room temperature before pyridine (0.1 ml, analytical grade) was added.

3.2.2.6.2 Silylation

Hexamethyldisilazane [HMDS] (0.2 ml, derivatisation grade) and trifluoroacetic acid (3 drops, analytical grade) were added to the samples, which were then shaken vigorously for 30 seconds and allowed to stand for 1 hour prior to analysis.

3.2.3 Analysis

3.2.3.1 Method A

This method was employed for the analysis of samples prepared using method 1, as described in section 3.2.2.1.

3.2.3.1.1 Instrumentation

A *5890 Series II Gas Chromatograph* (Hewlett Packard) fitted with a quadrupole mass selective detector was used. The detector temperature and the injector temperature were set at 280 °C and 250 °C, respectively. The column was a *DB-5* (5 % phenyl siloxane, length 30 m; ID 0.25 mm; film 0.25 microns, Fisher Scientific) and helium was used as the carrier gas (pressure 2.5 psi). Programme control and data processing were achieved using an *MS-Chemstation* with the *G1034B* software package (both Hewlett Packard).

3.2.3.1.2 Methodology

Separation and analysis were achieved using GC-MS. A completely splitless injection (12 minute solvent delay) of the sample (1 μl) was made onto the column. The 43 minute temperature programme had an initial setting of 100 °C for 2 minutes and was then ramped at a rate of 6 °C min^{-1} to a final temperature of 285 °C, which was held for 10 minutes. The scan parameters for the mass selective detector ranged between a minimum of 25 and a maximum of 650 atomic mass units.

3.2.3.2 Method B

This method was employed for the analysis of samples prepared using methods 1 to 5, as described in sections 3.2.2.1 to 3.2.2.5.

3.2.3.2.1 Instrumentation

A *5300 Mega Series Gas Chromatograph* (Carlo Erba Strumentazione) fitted with a flame ionisation detector was used. The detector temperature was 280 °C and the injector temperature was set at 250 °C. The column was a *DB*-5 (length 30 m; ID 0.25 mm; film 0.25 microns, Fisher Scientific) and nitrogen was used as the carrier gas (pressure 0.5 kg cm^{-2}). Data processing was achieved using the *Peak Simple* software package.

3.2.3.2.2 Methodology

Separation and analysis were achieved using GC. Sample (1 µl) was injected (split ratio 1:5) onto the column. The 43 minute temperature programme had an initial setting of 100 °C for 2 minutes and was then ramped, at a rate of 6 °C min^{-1}, to a final temperature of 285 °C, which was held for 10 minutes.

3.2.3.3 Method C

This method was employed for the analysis of samples prepared using methods 1 to 5, as described in sections 3.2.2.1 to 3.2.2.5.

3.2.2.3.1 Instrumentation

A *5300 Mega Series Gas Chromatograph* (Carlo Erba Strumentazione) fitted with a flame ionisation detector was used. The detector temperature was 280 °C and the injector temperature was 250 °C. The column was a *DB-17 HT* (50 % phenyl, 50 % methyl polysiloxane, length 30 m; ID 0.25 mm; film 0.15 microns, Fisher Scientific) and nitrogen was used as the carrier gas (pressure 0.5 kg cm^{-2}). Data processing was achieved using the *Peak Simple* software package.

3.2.2.3.2 Methodology

Separation and analysis were achieved using GC. Sample (1 µl) was injected (split ratio 1:5) onto the column. The 45 minute temperature

programme had an initial setting of 120 °C, held for 2 minutes and was then ramped, at a rate of 5 °C min^{-1}, to a final temperature of 260 °C, which was held for 15 minutes.

3.2.3.4 Method D

This method was employed for the analysis of samples prepared using methods 4 to 6, as described in sections 3.2.2.4 to 3.2.2.6. The samples of standard gum media and monosaccharides were diluted prior to analysis: 5 drops of sample were diluted with pyridine (1 ml, analytical grade) and thoroughly mixed.

3.2.3.4.1 Instrumentation

A *5890 Series II Gas Chromatograph* (Hewlett Packard) fitted with a quadrupole mass selective detector was used. The detector temperature was set at 280 °C and the injector temperature at 250 °C. The column was a *DB-5* (length 30 m; ID 0.25 mm; film 0.25 microns, Fisher Scientific) and helium was used as the carrier gas (pressure 2.5 psi). Programme control and data processing were achieved using an *MS-Chemstation* with the *G1034B* software package (both Hewlett Packard).

3.2.3.4.2 Methodology

Separation and analysis were achieved using GC-MS. A completely splitless injection (12 minute solvent delay) of sample (1 µl) was made onto the column. The 43 minute temperature programme had an initial setting of

100 °C, held for 2 minutes and was then ramped, at a rate of 6 °C per minute, to a final temperature of 285 °C, which was held for 10 minutes. Detection was achieved *via* selected ion monitoring (SIM) - operating parameters were set as follows:

dwell time per ion - 100 milliseconds

ions monitored - 73.00, 147.00, 204.00, 217.00 amu.

The ion at 73.00 amu corresponds to the TMS group from the silylating agent itself, whilst the other ions, silylated ion fragments of the monosaccharides, were selected as those most prevalent in the mass spectra of the individual standard monosaccharides.

CHAPTER 4

Results

4.1 Standard Media Samples

4.1.1 Proteinaceous Media

There are a number of important ratios which are useful in the identification of unknown proteinaceous binding media. Hydroxyproline is found almost exclusively in collagen, though trace amounts may be found in other proteinaceous materials, and its appearance indicates the presence of animal glue, even as a component in a complex mixture. It is, therefore, possible to say that if levels of hydroxyproline are greater than 2 %, the sample contains collagen-based glue. A number of specific amino acid ratios confirm the presence of an animal glue:[55]

- a high level of glycine, around 30 %,
- a 1:8 ratio of serine:glycine,
- an approximately 1:1 ratio of alanine:proline, and
- low levels of essential amino acids, such as valine, phenylalanine and leucine.

Tables 4.0 to 4.5 show the relative percentage amino acid compositions obtained for a variety of proteinaceous glue media: those in table 4.0 were published by Mills and White,[111] whilst those in tables 4.1 to 4.5 were obtained in the course of this particular research programme.

Casein has been reported as being characterised by the following typical levels of certain amino acids:[55]

- high levels of glutamic acid (no less than 15 %) and proline (greater than 8 %),
- less than 7 % aspartic acid, and
- an even balance of essential amino acids, *e.g.* leucine, alanine and methionine, of between 3 and 10 %.

Amino Acid	Gelatine	Casein	Egg White	Egg Yolk
Hydroxy-proline	7.4	0.0	0.0	0.0
Serine	4.0	4.0	5.8	9.1
Aspartic Acid	5.0	6.1	10.5	11.5
Glutamic Acid	9.7	20.2	13.9	15.0
Arginine/ Threonine	8.2/2.2	4.0/2.7	6.8/3.7	5.5/5.6
Glycine	24.7	1.7	3.6	3.5
Alanine	10.1	2.7	6.3	5.6
Proline	13.0	13.2	4.5	4.5
Valine	2.2	7.2	8.3	6.4
Phenylalanine	1.6	5.1	5.2	3.9
Leucine	3.7	9.0	10.3	9.2
Isoleucine	1.2	6.0	6.2	5.1
Lysine	4.1	6.7	8.0	5.7
Methionine	1.4	2.3	1.2	2.3
Histidine	1.5	3.6	2.4	2.4
Tyrosine	0.0	5.5	1.4	2.8
½ Cystine	0.0	0.0	1.9	1.9

Table 4.0: *Published Amino Acid Compositions of Proteinaceous Materials Used as Binding Media and Adhesives (Mills and White)*[111]

Egg proteins may be distinguished from casein by the much lower levels of proline observed.[37] The approximately 2:1 serine:glycine ratio and slightly lower alanine:proline ratio seen in egg proteins can also be used for purposes of identification, but they seem to be subject to some variation. The weakest distinction is between egg yolk and egg albumin, which relies on the slight differences between levels of threonine and serine – the even

proportions of essential amino acids, which make the egg a good source of dietary protein, also make differentiation between the white and yolk difficult. Whilst aspartic acid levels in both are greater than 8 %, the threonine and serine levels in yolk are typically 5-8 % and 9-12 % respectively, compared with 3-4 % threonine and 7-8 % serine in albumin.[37]

As mentioned previously, Mills and White[111] published a table of amino acid compositions of proteins commonly used as binding media and adhesives – the original results were taken from Keck and Peters' investigations[37] and converted to weight percent of total (table 4.0 shows these compositions).

However, the results observed in the course of this study have shown slight differences in the average relative amino acid composition of samples of casein and egg media from those previously reported in the literature. Possible reasons for these slight differences could be the variations in experimental methodology, *i.e.* derivatisation technique, and instrumentation - the efficiency of reactions and detection will be different for each method of sample preparation and analysis. The experimental results are shown in tables 4.6 to 4.8 and, since they have been obtained under the same experimental conditions as those of the samples removed from works of art, they are the ones that have been used for identification purposes.

Repeatability studies were performed. At least six derivatised samples of a single larger sample of each standard proteinaceous medium were prepared and each one was injected twice, to check the injection itself. A selection of different glue media from a variety of suppliers was investigated, in addition to egg and casein media. Tables 4.1 to 4.8 show the amino acid

composition and the relative standard deviations for the peak area of each amino acid identified in the standard samples of proteinaceous media.

Casein was obtained from three different suppliers in order that differences in the composition of the casein obtained from various sources could be investigated. Ideally, at least 6 different sources of casein should have been used in order to obtain a statistically meaningful average, but limitations in funding precluded this. The amino acid composition of the casein samples is shown in table 4.6, whilst table 4.7 shows the average over the three sources.

Table 4.8 shows the amino acid composition of egg albumin (glair) and egg yolk (both from hens). The samples of egg yolk medium which had been subjected to artificial ageing had compositions almost identical to those which were unaged, so it was concluded that, with the exception of methionine which disappears during the ageing process (probably due to cross-linking), the amino acids remain stable.

Grzywacz[112] investigated the amino acid composition of eggs from different species and locations: she found that whilst it was not possible to distinguish between eggs from different locations, it may be possible to differentiate between eggs from hens, ducks or geese. Slight differences in certain amino acid ratios for a whole egg binder could be used to determine the species of origin. However her suggestion that, as many hens are now fed hormones, the results obtained may not be directly applicable to aged samples from works of art must be borne in mind.

Amino Acid	Animal Glue Average % (n = 9)	Animal Glue RSD % (n = 9)	Rabbit Skin Glue Average % (n = 6)	Rabbit Skin Glue RSD % (n = 6)
Hydroxy-proline	10.49	1.61	16.08	1.95
Serine	3.07	1.69	1.48	0.09
Aspartic Acid	4.96	2.52	3.29	0.36
Glutamic Acid	6.95	2.35	2.79	0.34
Arginine/ Threonine	2.52	1.57	1.70	0.18
Glycine	34.22	3.77	33.88	3.13
Alanine	9.94	1.88	7.66	0.48
Proline	13.25	2.69	16.24	1.78
Valine	1.69	0.79	2.63	0.09
Phenylalanine	2.77	1.89	2.13	0.11
Leucine	2.39	1.48	1.99	1.17
Isoleucine	2.92	1.24	2.67	1.30
Lysine	2.75	1.59	2.89	0.42

Table 4.1: *Average Relative Percentage Composition and Relative Standard Deviation for Peak Area – Animal Glue & Rabbit Skin Glue*

Amino Acid	Isinglass Average % (n = 6)	Isinglass RSD % (n = 6)	Fish Glue Average % (n = 6)	Fish Glue RSD % (n = 6)
Hydroxy-proline	9.20	3.39	11.76	3.26
Serine	1.63	0.88	2.95	0.93
Aspartic Acid	5.11	2.06	4.93	0.50
Glutamic Acid	3.96	1.73	7.42	0.29
Arginine/ Threonine	2.43	1.09	2.16	0.22
Glycine	34.34	10.39	35.39	2.13
Alanine	5.21	1.80	9.59	2.77
Proline	6.64	3.49	12.29	3.17
Valine	3.66	4.40	1.44	0.27
Phenylalanine	1.36	0.81	1.57	0.22
Tryptophan	1.05	0.47	-	-
Leucine	1.05	0.563	1.20	0.202
Isoleucine	1.31	0.500	2.44	1.260
Lysine	3.16	1.113	3.35	1.089

Table 4.2: *Average Relative Percentage Composition and Relative Standard Deviation for Peak Area – Isinglass & Fish Glue*

Amino Acid	Bone Glue Average % (n = 6)	Bone Glue RSD % (n = 6)	Parchment Average % (n = 6)	Parchment RSD % (n = 6)
Hydroxy-proline	18.62	5.90	25.59	5.35
Serine	3.36 in total	0.70 in total	0.47	0.08
Aspartic Acid			2.40	0.28
Glutamic Acid	3.80	0.92	1.82	0.20
Arginine/ Threonine	1.85	0.36	1.99	0.19
Glycine	32.74	5.58	28.01	1.84
Alanine	5.80	2.17	2.89	0.70
Proline	19.47	5.49	23.39	1.37
Valine	0.91	0.66	0.25	0.08
Phenylalanine	1.70	0.76	0.88	0.37
Tryptophan	1.64	0.48	1.36	0.49
Leucine	1.25	0.92	0.36	0.07
Isoleucine	1.99	0.64	0.98	0.19
Lysine	2.27	0.91	0.37	0.02

Table 4.3: *Average Relative Percentage Composition and Relative Standard Deviation for Peak Area – Bone Glue & Parchment for Glue Making*

Amino Acid	High Strength Skin Glue Average % (n = 6)	High Strength Skin Glue RSD % (n = 6)	Gelatine Sheet Average % (n = 6)	Gelatine Sheet RSD % (n = 6)
Hydroxy-proline	29.67	5.40	28.82	6.80
Serine	2.30 in total	0.65 in total	2.43 in total	0.39 in total
Aspartic Acid				
Glutamic Acid	2.00	0.21	4.02	0.77
Arginine/ Threonine	1.26	0.25	1.56	0.30
Glycine	30.38	1.72	28.84	3.66
Alanine	2.33	0.42	3.02	1.39
Proline	26.34	1.58	23.93	4.21
Valine	0.36	0.04	0.35	0.07
Phenylalanine	0.82	0.26	0.91	0.56
Tryptophan	1.29	0.27	0.98	0.48
Leucine	0.42	0.06	0.49	0.26
Isoleucine	0.91	0.06	1.01	0.67
Lysine	0.48	0.05	1.04	0.78

Table 4.4: *Average Relative Percentage Composition and Relative Standard Deviation for Peak Area – High Strength Skin Glue & Gelatine Sheet*

Amino Acid	Overall Average % (n = 51)	Overall RSD % (n = 51)
Hydroxyproline	18.78	7.77
Serine	1.85	0.95
Aspartic Acid	3.54	1.17
Glutamic Acid	4.10	1.96
Arginine/Threonine	1.93	0.40
Glycine	32.22	2.60
Alanine	5.81	2.82
Proline	17.70	6.35
Valine	1.41	1.14
Phenylalanine	1.52	0.64
Tryptophan	1.26	0.24
Leucine	1.14	0.69
Isoleucine	1.78	0.77
Lysine	2.04	1.14

Table 4.5: *Overall Average Relative Percentage Composition and Relative Standard Deviation for Peak Area - Collagen-Based Glues*

Amino Acid	Casein 1 Avge. % (n = 7)	Casein 1 RSD % (n = 7)	Casein 2 Avge. % (n = 6)	Casein 2 RSD % (n = 6)	Casein 3 Avge. % (n = 6)	Casein 3 RSD % (n = 6)
Serine	2.62	1.43	1.39	0.23	4.15	0.42
Aspartic Acid	9.81	0.27	10.76	1.51	8.78	0.18
Glutamic Acid	13.59	3.36	8.67	1.51	19.36	0.55
Arginine/ Threonine	3.32	0.28	10.52	1.94	3.99	0.21
Glycine	8.37	2.12	8.17	1.71	5.44	1.13
Alanine	5.78	1.04	3.63	0.46	3.34	0.23
Proline	7.28	1.83	9.02	1.47	10.96	1.32
Valine	9.33	0.56	4.89	0.69	8.38	0.25
Phenylalanine	8.41	2.28	10.13	0.62	4.96	0.42
Leucine	11.86	1.56	11.94	2.34	10.30	0.49
Isoleucine	5.97	0.55	5.41	0.99	5.98	0.47
Lysine	5.12	1.30	4.21	3.17	3.88	0.44

Table 4.6: *Average Relative Amino Acid Composition - Casein*

Amino Acid	Overall Average %	Overall RSD %
Serine	2.72	1.13
Aspartic Acid	9.78	0.81
Glutamic Acid	13.87	4.37
Arginine/Threonine	5.94	3.25
Glycine	7.33	1.34
Alanine	4.25	1.09
Proline	9.09	1.50
Valine	7.53	1.91
Phenylalanine	7.83	2.15
Leucine	11.37	0.76
Isoleucine	5.79	0.27
Lysine	4.40	0.52

Table 4.7: *Overall Average Relative Percentage Composition and Relative Standard Deviation for Peak Area - Casein*

Amino Acid	Egg Albumin Average % (n = 7)	Egg Albumin RSD % (n = 7)	Egg Yolk Average % (n = 15)	Egg Yolk RSD % (n = 15)
Serine	14.66 in total	1.36 in total	8.68	2.42
Aspartic Acid			13.58	1.23
Glutamic Acid	7.16	3.26	13.07	2.18
Arginine/ Threonine	8.50	2.50	6.47	0.75
Glycine	10.54	4.41	7.75	1.35
Alanine	6.90	1.95	9.10	1.67
Proline	4.20	0.97	5.78	1.04
Valine	5.73	0.93	6.60	1.03
Phenylalanine	9.32	0.73	4.22	0.76
Leucine	9.76	1.74	14.59 in total	0.88 in total
Isoleucine	5.00	0.80		
Lysine	4.04	2.31	8.20	2.63

Table 4.8: *Average Relative Percentage Composition and Relative Standard Deviation for Peak Area – Egg Albumin and Egg Yolk*

Figures 4.1 to 4.3 show the chromatograms obtained for a standard mixture of amino acids (for peak identification purposes) and standard samples of animal glue, casein, egg yolk and egg albumin media.

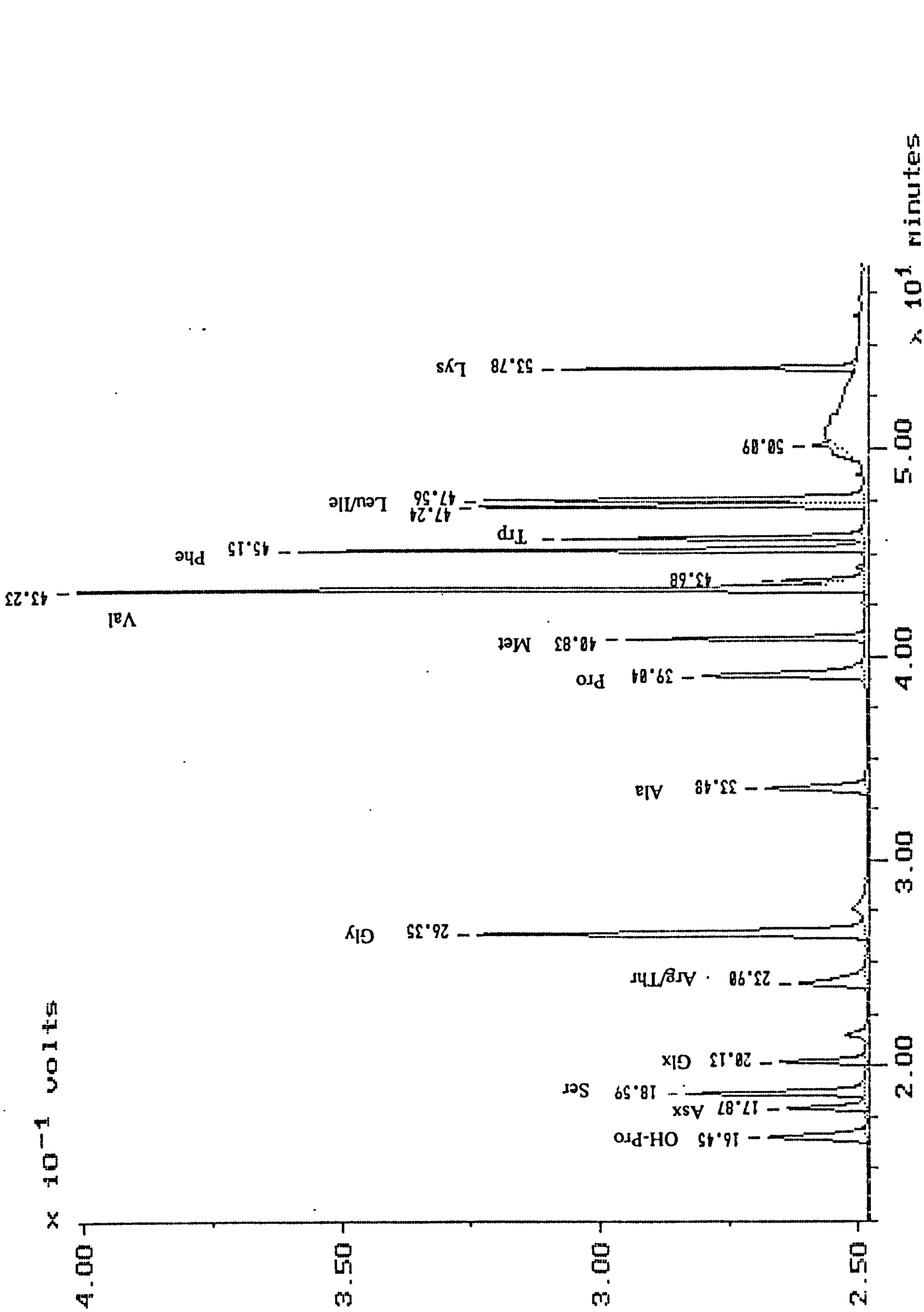


Figure 4.1: *Chromatogram for Standard Amino Acid Mixture*

(for Peak Identification)

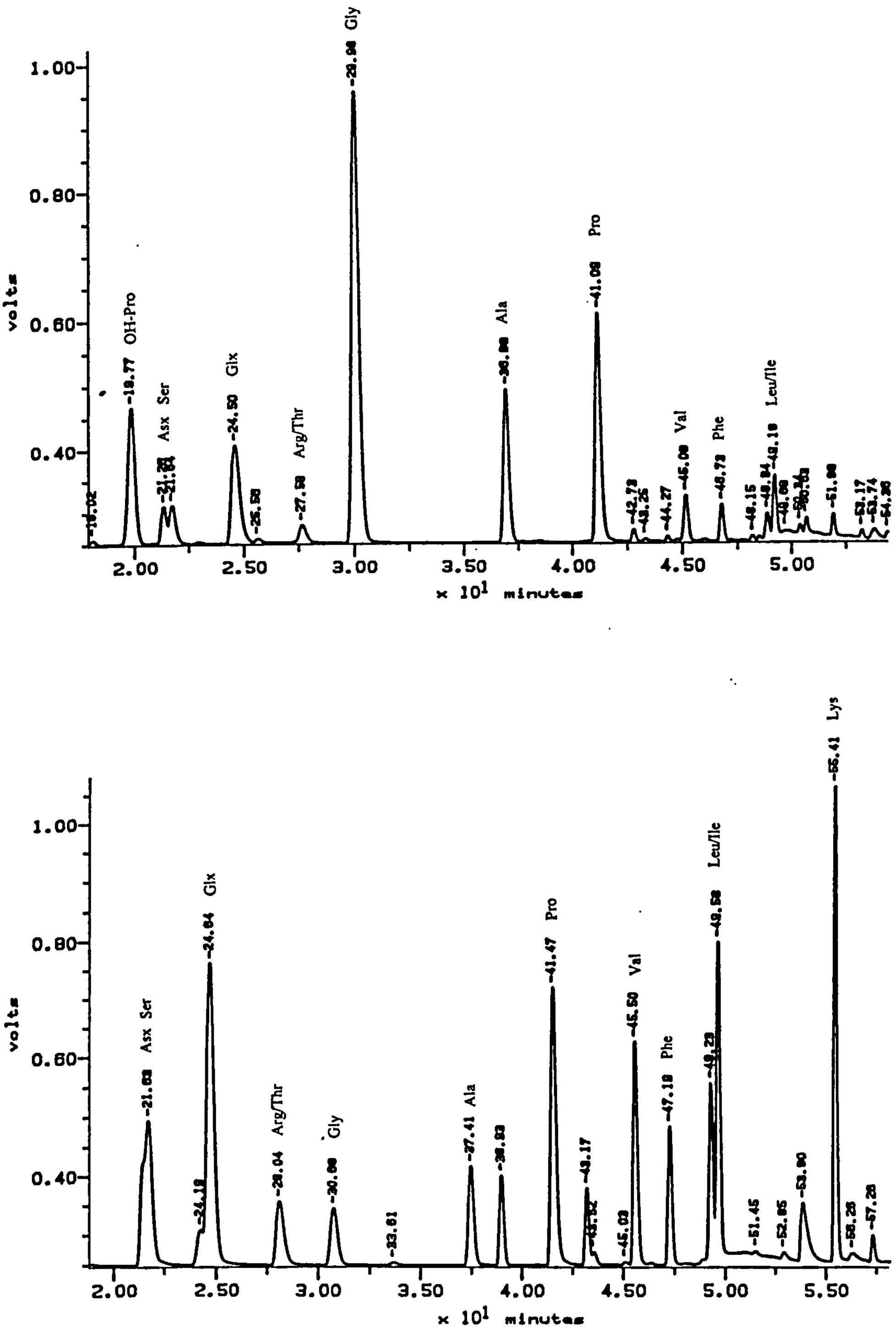


Figure 4.2: *Chromatograms for Standard Animal Glue and Casein Samples*

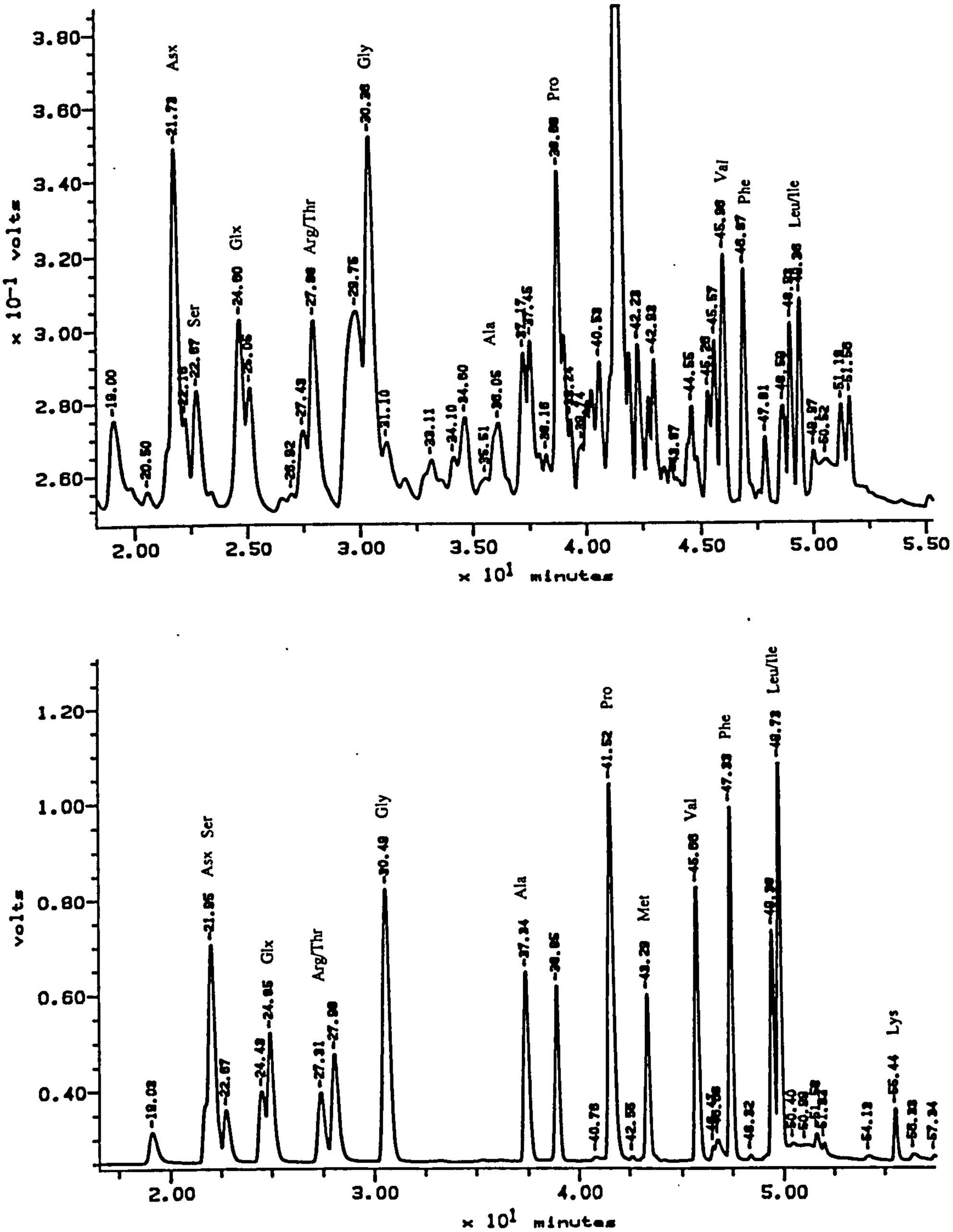


Figure 4.3: *Chromatograms for Standard Hens' Egg Yolk and Egg Albumin Samples*

4.1.2 Gum Media

The six different methods of sample preparation were investigated with varying degrees of success. Sample preparation methods 1 to 4 (chapter 3, sections 3.2.2.1 to 3.2.2.4) proved not to be reproducible, peaks appearing and disappearing randomly over series of samples, whilst analytical method A to C (chapter 3, sections 3.2.3.1 to 3.2.3.3) were insufficiently sensitive to detect the extremely low levels of gum material in the minute samples removed from works of art.

The initial investigations using standard monosaccharide samples, employing method 1 for sample preparation and method A for analysis, gave results which did appear to be useful at first. The retention data obtained is shown in table 4.9.

TMS Oxime Derivative	t_r peak 1 /min	t_r peak 2 /min	$t_r2 - t_r1$	Peak area ratio (1:2)	Main Ions for GC-MS
Arabinose	18.92	19.06	0.14	-	-
Rhamnose	20.13	20.37	0.24	-	-
Xylose	18.91	19.27	0.36	-	-
Fucose	20.22	20.56	0.34	-	-
Mannose	23.11	23.55	0.44	5.94	**73, 147 ,205, 319**
Glucuronic Acid	23.10	23.53	0.43	4.52	**73, 147, 218**

Table 4.9: *Retention Data for a Selection of Standard Monosaccharide TMS Oxime Derivatives*

Two peaks were seen for each structure, corresponding to the two isomeric forms (Z and E) of the TMS oxime derivatives. Structurally the monosaccharides are very similar, hence it is inevitable that their retention times will also be very similar: this leads to problems with their identification. By calculating the difference between the retention times of the two peaks, it

was possible to differentiate between arabinose and xylose (t_r2 - t_r1 xylose > t_r2 - t_r1 arabinose) and rhamnose and fucose (t_r2 - t_r1 fucose > t_r2 - t_r1 rhamnose). However, this data was not sufficient to distinguish between glucuronic acid and mannose: it was necessary to calculate the peak area ratio for peak 1:peak2 and to look at the main ions present in the mass spectrum in order to ascertain the identity of the monosaccharide derivative. Mannose had a much higher peak 1:peak 2 area ratio than glucuronic acid, whilst there were a number of differences in the mass spectra of the two sugars which ultimately facilitated their identification. Since the actual masses of samples were unknown the analytical method was purely qualitative, peak height ratios being calculated on the assumption that the derivatisation process was repeatable.

A selection of standard gum media were prepared and analysed using the same method; the retention data obtained for the individual monosaccharide derivatives was then used to identify the major sugar components in the gum samples. Table 4.10 shows the main sugar components of the gums, based on the retention data in table 4.9.

Standard Gum Sample	Major Sugar Components
Arabic	Arabinose, rhamnose, glucuronic acid
Tragacanth	Arabinose
Guar	Mannose
Ghatti	Mannose, Arabinose
Karaya	Rhamnose

Table 4.10: *Major Sugar Components of Standard Gum Samples*

From the results obtained using relatively large samples of standard media it appeared that it would be possible to identify gum materials qualitatively by GC-MS, from their sugar compositions.

Hydrolysis was most efficiently and repeatably achieved using undiluted trifluoroacetic acid (method 3, section 3.2.2.3). The presence of water in the acid solutions used for hydrolysis in methods 1 and 2 (sections 3.2.2.1 and 3.2.2.2) led to incomplete silylation where TSIM was the silylating agent.

Oxime formation also appeared to be subject to variation, though less complicated chromatograms were obtained for the oxime derivatives. Despite this obvious advantage, the oxime route was abandoned as it added an extra step to sample preparation, increasing the volume and therefore needlessly diluting the already weak sample solutions.

Silylation using hexamethyldisilazane in the presence of a trifluoroacteic acid catalyst is unaffected by moisture,[113] the reaction accommodating water levels up to around 50 % of the sample mass, thus resulting in more repeatable silylation. This, coupled with the ease of silylation of the sugar OH groups, led to the selection of HMDS as the derivatising agent instead of TSIM.

Analytical methods A to C did not provide sufficient sensitivity for "real" samples, though resolution of the detectable peaks was good. The split-splitless injections used in methods B and C reduced the amount of sample actually going onto the column and, since the samples contained very small amounts of gum material, only the major sugar components were detectable. Method A used a completely splitless injection, so all the sample

was injected onto the column, but was still not capable of detecting minute levels of monosaccharide derivatives. For this reason method D, differing only from method A in its detection technique, was employed for sample analysis. Selected ion monitoring (SIM) allows the study of a limited number of ions so more data/mass can be collected, thereby increasing sensitivity. Method D facilitated the detection of even the smallest components present in the gum materials. Results obtained for the individual standard monosaccharides (using sample preparation method 5 and analytical method D) will be considered first.

Multiple peaks were seen for each sugar, corresponding to the different structural forms of the silylated derivatives: table 4.11 shows the retention data for the individual monosaccharides studied.

Silylated Monosaccharide Derivative	Retention Time of Major Component Peak (Minutes)	Retention Time of Minor Component Peak (Minutes)
Arabinose	16.18	16.28, 16.90, 17.43
Rhamnose	16.52	15.37, 17.85
Fucose	17.41, 18.19	16.53, 16.71, 18.29
Xylose	18.11	16.46, 16.70, 19.12
Mannose	20.02	17.55, 21.79
Galactose	20.99	20.25, 21.75
Glucose	21.57, 23.08	18.00, 19.50, 21.25
Galacturonic Acid	21.01, 22.72	15.04, 22.27, 23.84
Glucuronic Acid	20.38	16.52, 20.24, 22.87

Table 4.11: *Retention Data for Standard Silylated Monosaccharides*

Using the retention data in table 4.11, the sugar components of each of the standard gum media samples were identified. As the analysis was qualitative rather than quantitative, the actual percentage composition of the samples was not determined. However, since the derivatisation process

appeared to be repeatable, it was possible to assess the levels of a component relative to each of the others in gum material. The composition of the gums is shown in table 4.12 - the relative level of each component is expressed by the terms "major" (main component), "moderate" (reasonably abundant component) and "minor" (lesser component) and "trace" (least significant component).

Chromatograms obtained for unaged and artificially aged standard gum media were almost identical, the sugar composition remaining virtually unchanged after thermal/light ageing: samples of suspected gum media removed from works of art have been naturally ageing for many years, thus the artificially aged gums were used as standards for identification purposes.

Figures 4.4 to 4.6 show the chromatograms obtained for samples of aged standard gum media (arabic, tragacanth, cherry, karaya, guar, ghatti, locust bean) and other carbohydrate materials known to have been used by artists (rice starch, honey, egg albumin and brown sugar).

Only gum tragacanth contains xylose and fucose in significant levels (around 13 % and 4.5 % respectively).[78] These two sugars are sometimes present as very minor components of gum ghatti[78] (up to 1.7 % and 2.3 % respectively) and xylose is found in low levels (around 4-6 %) without fucose in cherry gum.[92] Reasonable levels of xylose, plus a small amount of fucose, in a sample would therefore indicate the presence of gum tragacanth.

Arabinose is virtually undetectable in gum karaya , locust bean gum and guar gum, whilst mannose is the major component of both guar gum and locust bean gum.[78] Hence, if a sample contained no arabinose or mannose, karaya gum would be the expected medium.

Sugar Component	Gum Arabic	Gum Tragacanth	Cherry Gum	Gum Ghatti	Karaya Gum	Guar Gum	Locust Bean Gum
Arabinose	Major	Major	Major	Major		Trace	Trace
Rhamnose	Minor			Minor	Moderate		
Galactose	Major	Moderate	Moderate	Major	Major	Moderate	Moderate
Mannose			Trace			Major	Major
Fucose		Minor					
Xylose		Moderate	Minor				
Glucose		Trace				Trace	Trace
Galacturonic Acid		Moderate			Major		
Glucuronic Acid	Moderate		Minor	Trace	Minor		
Unknown	Major	Moderate	Moderate	Minor	Major	Minor	Minor

Table 4.12: *Sugar Components Identified in Standard Gum Media Samples*

Very high levels of mannose in the absence of arabinose would suggest either a locust bean gum or guar gum medium.

Gum arabic is indicated by the presence of relatively high levels of arabinose and rhamnose (approximately 18 % and 11 % respectively), whilst gums tragacanth and ghatti contain larger amounts of arabinose (around 22 % and 32 %) but much lower levels of rhamnose (up to 6 % and 6.5 % respectively).[78] Cherry gum contains a very large amount of arabinose (around 55 %) with a negligible amount of rhamnose.[92]

The uronic acids are not found in all of the common gum media studied: galacturonic acid is found only in gums tragacanth and karaya and glucuronic acid is a component of gums arabic, ghatti, karaya and cherry.[78,92]

All the samples of suspected gum media removed from works of art were prepared for analysis using method 6 (section 3.2.2.6) and analysed *via* method D (section 3.2.3.4).

The following sections contain tables of results for the amino acid and sugar compositions of proteinaceous and gum media removed from works of art. The composition of proteins is expressed in terms of the relative percentages of each amino acid, whilst the monosaccharide composition of the gums is represented by the number of √s – the more significant the component, the greater the number of √s (*i.e.* the major component is indicated by 4 √s, whilst the least significant component is indicated by only 1 √).

4.2 Selected Paintings from the Conservation Department, University of Northumbria at Newcastle

Students from the University's MA course in Conservation of Fine Art are given a conservation project in their second year: part of the project involves the identification of pigments and binding media, hence samples removed from selected project works were used in order to test the method developed for the analysis of proteinaceous media.

Samples from paintings on canvas were submitted for protein analysis: a sample of lining adhesive was removed from *Portrait of Sir James Lowther (1736-1802)* [c. 1755; Thomas Hodson (attributed), oil on canvas] (MA1) and upon analysis, the adhesive was found to be animal glue.

Works on paper were also sampled for analysis. Carbon black paint (MA 2), suspected of containing an animal glue or gum medium, was removed from an 18th century French watercolour map of Europe (c. 1788; cartographer, Duke Angoulême). Protein analysis did indeed reveal the use of animal glue in the paint, though at the time of analysis there was no suitable method available to confirm the additional presence of a gum medium.

A sample of suspected animal glue (MA 3) was removed from *Portrait of a Gentleman* (artist unknown), a work thought to be early 19th century Chinese art for the export market. The amino acid composition was found to correspond with that of a standard collagen based glue, thus an animal glue medium was confirmed.

Finally, samples of brown paint (MA 4) from the border and cream paint (MA 5) used for the lettering were removed from a railway poster. As

before, the suspected use of an animal glue medium was confirmed by protein analysis. Table 4.13 shows the amino acid compositions of the samples from the MA student project works submitted for protein analysis. Chromatograms obtained from the analysis of these samples can be found in appendix 4.

Amino Acid	Relative % Composition				
	MA 1	MA 2	MA 3	MA 4	MA 5
OH-Pro	8.84	7.33	12.36	3.20	5.13
Ser	6.15	4.84	1.99	1.19	5.13
Asx	5.89	10.93	5.47	3.66	8.55
Glx	7.28	9.80	3.92	3.25	8.55
Arg/Thr	1.79	-	1.91	9.39	2.56
Gly	40.33	27.03	30.39	57.96	20.51
Ala	11.89	10.43	13.18	4.31	6.84
Pro	11.06	15.56	7.10	6.33	8.55
Val	1.24	4.58	3.05	3.91	22.22
Phe	2.50	4.90	7.46	-	-
Leu/Ile	2.04	4.60	9.19	4.27	-
Lys	0.99	-	3.97	-	-

Table 4.13: *Amino Acid Composition of Samples Removed from MA Conservation Student Project Works*

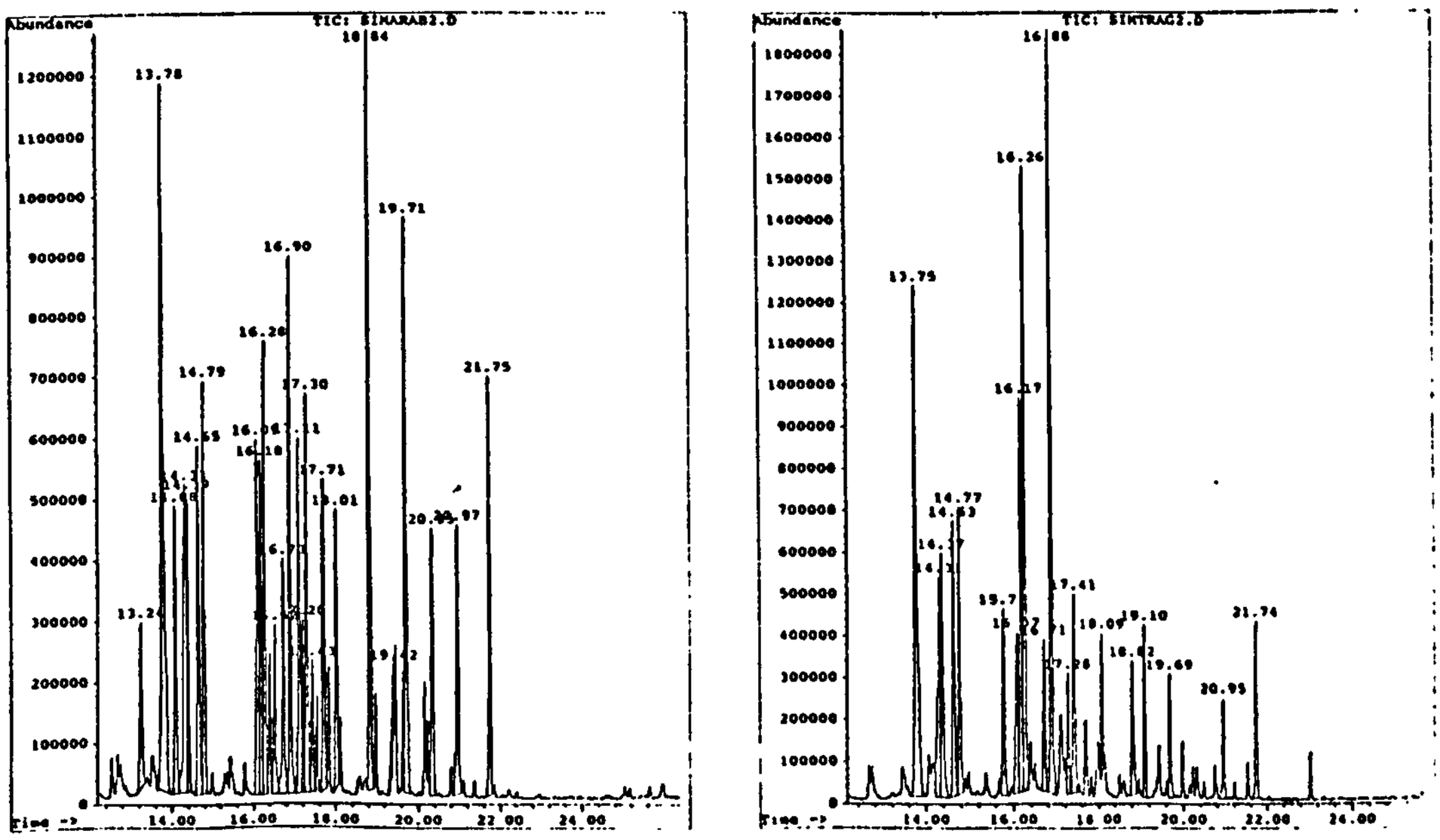
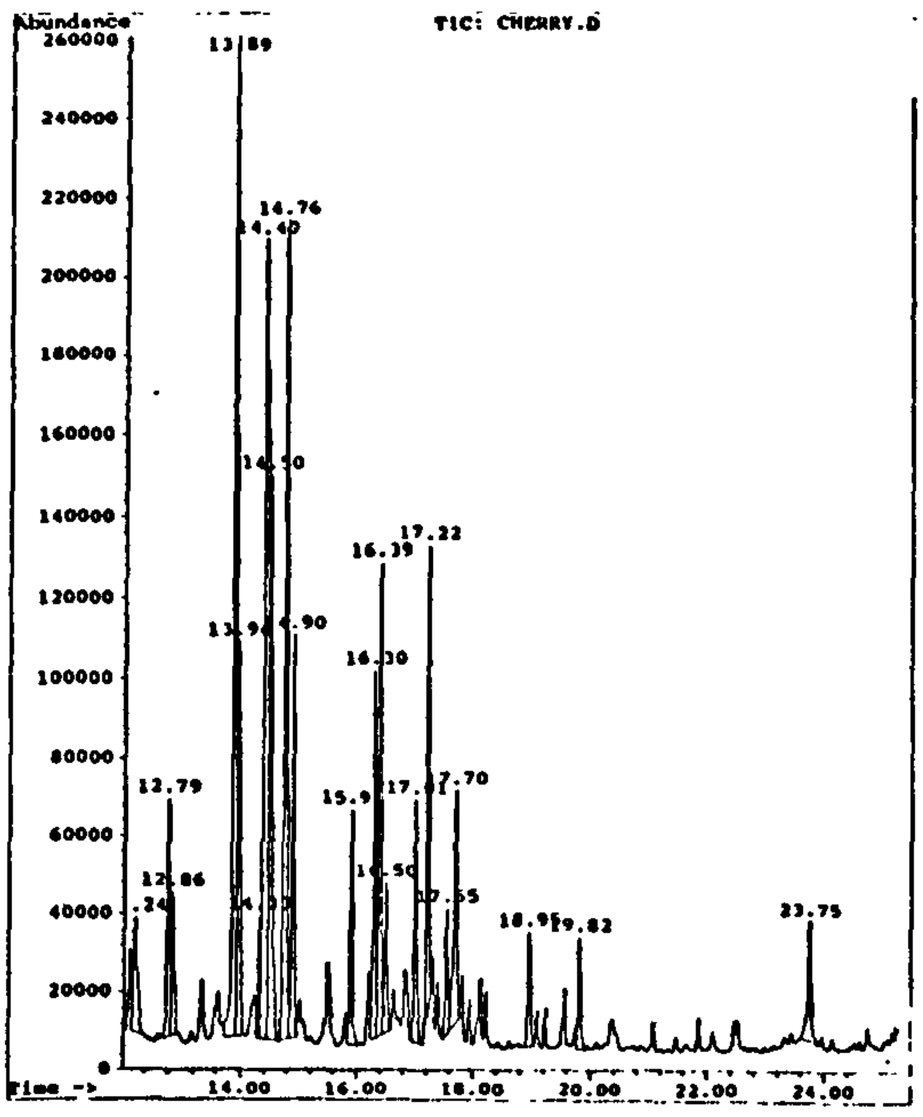
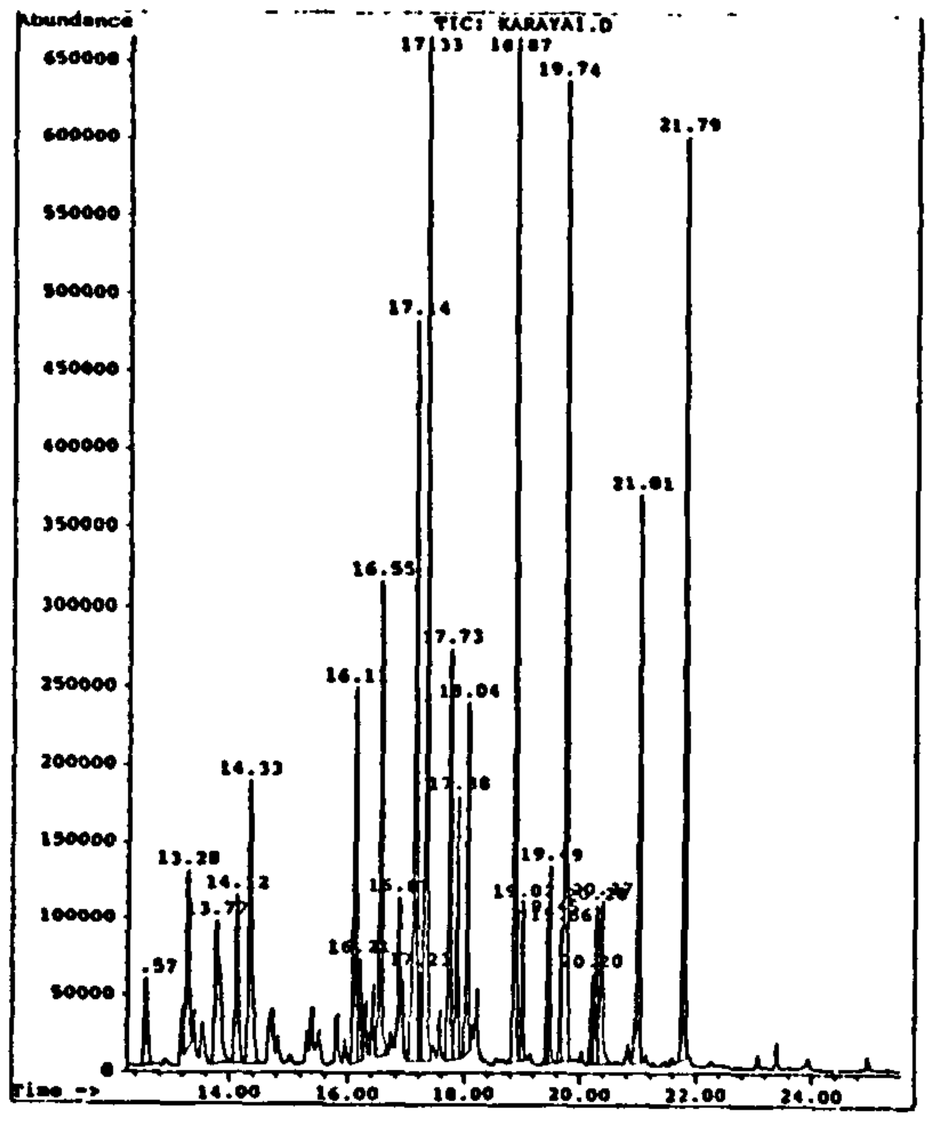
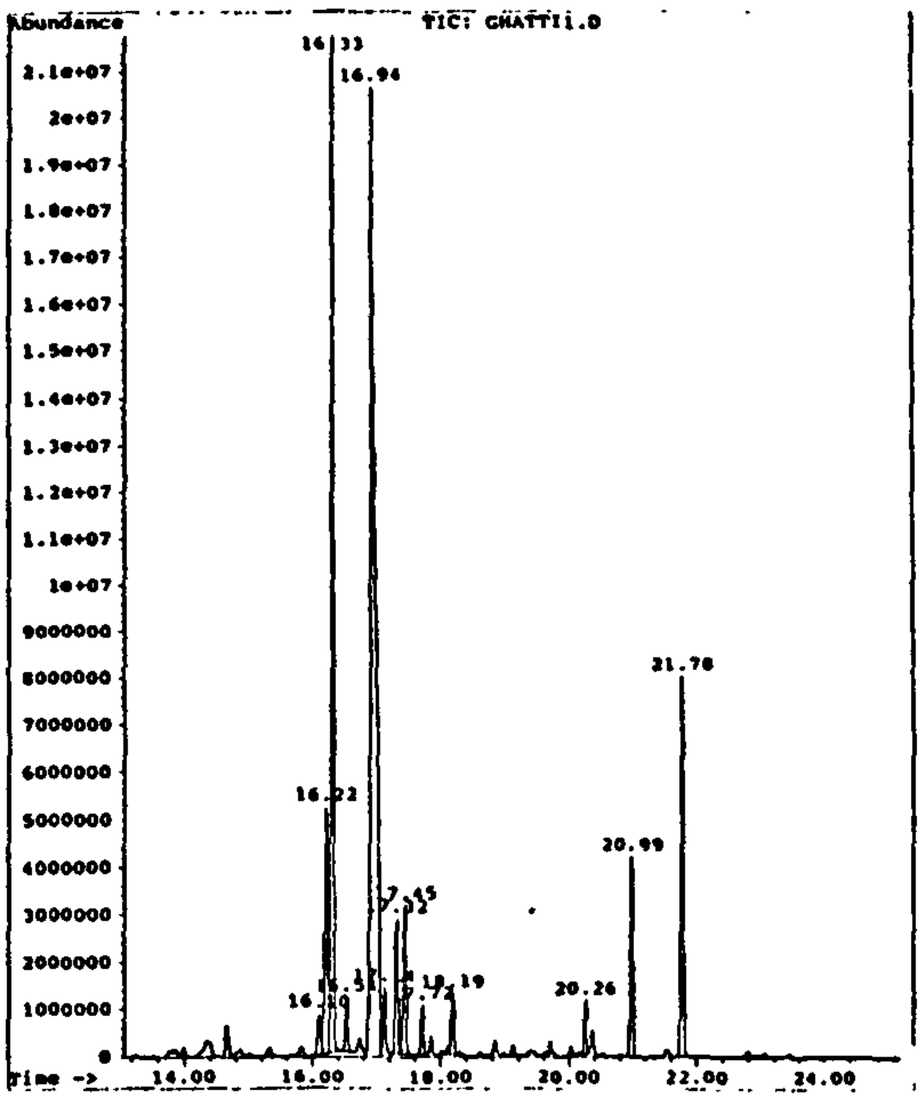


Figure 4.4: *Chromatograms for Standard Aged Samples of Gums Arabic and Tragacanth*







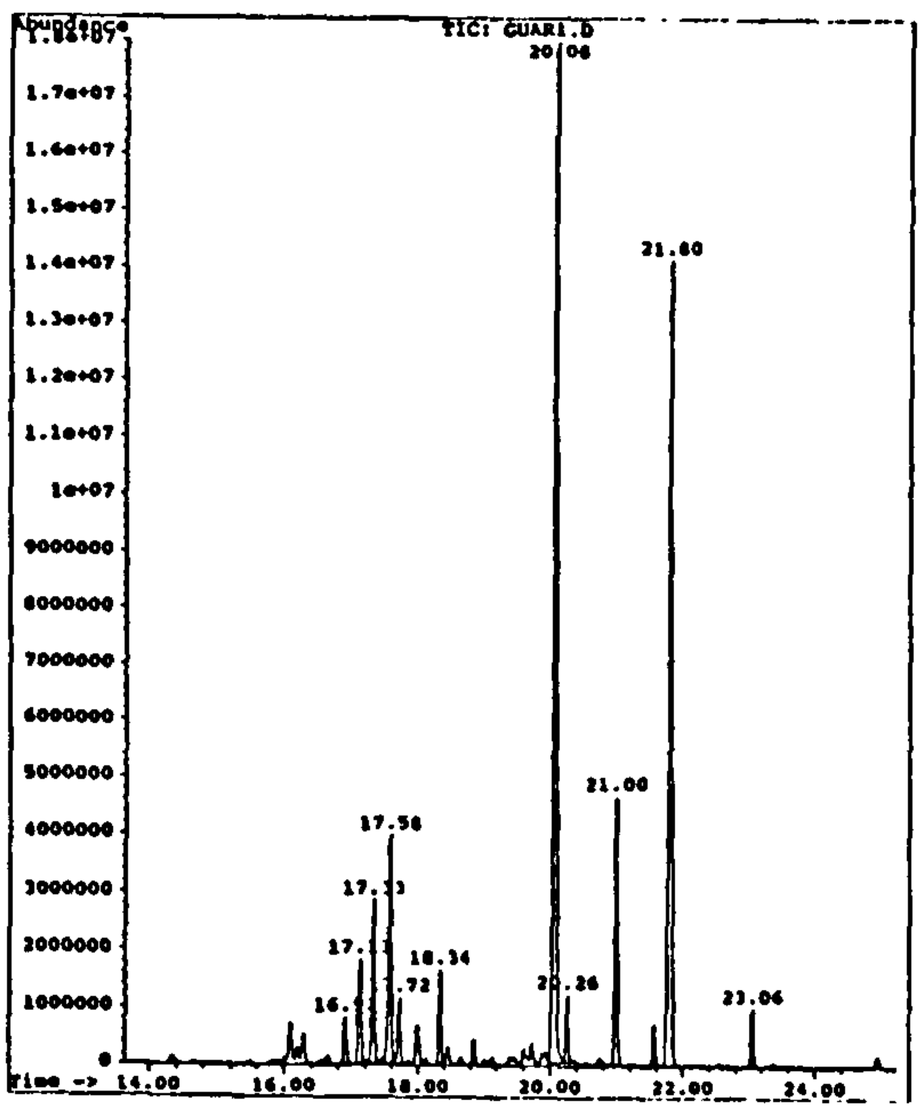


Figure 4.5: *Chromatograms of Standard Aged Samples of Gums Cherry, Karaya, Ghatti, and Guar*

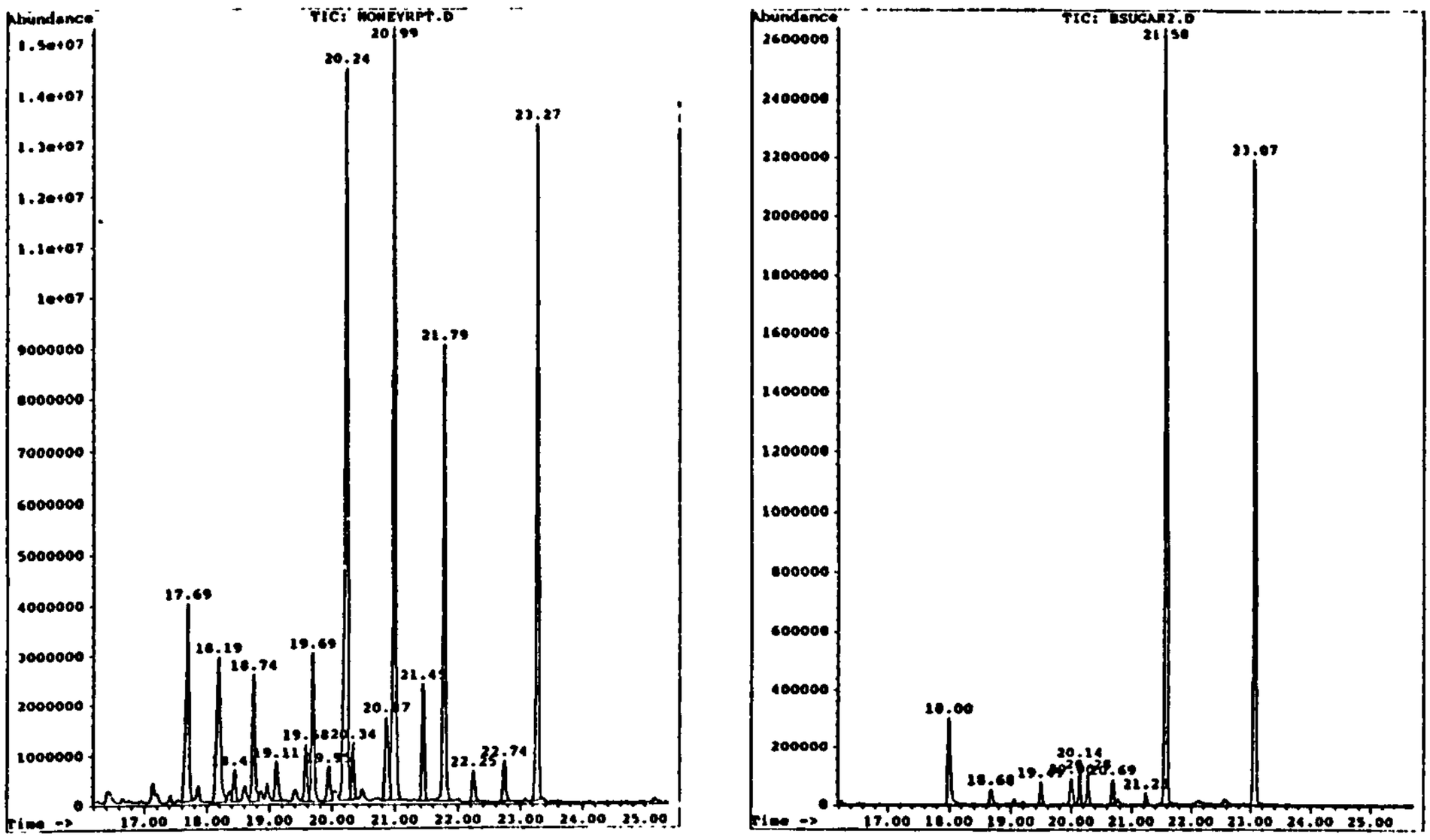


Figure 4.6: *Chromatograms for Standard Samples of Honey, Brown Sugar, Rice Starch and Egg Albumin*

4.3 Paintings from the Tate Gallery's Collection

RP-HPLC and GC chromatograms obtained from the analysis of samples removed from works from the Tate Gallery's collection can be found in appendix 4.

4.3.1 Works of William Blake (1757-1827)

The first painting studied was *The Body of Christ Borne to the Tomb* (Tate Gallery N01164, c. 1799-1800) [plate 1, appendix 3]. The work, described as tempera on canvas, has previously undergone some retouching/consolidation with gelatine, the nature of which is currently unexamined, and exhibits a reasonable amount of flaking/cracking. Samples of green paint, blue paint from the sky and priming were removed and stored in glass vials prior to protein/gum analysis.

Tables 4.14 and 4.15 show the amino acid and sugar compositions of the paint and priming samples.

Amino Acid Residue	% Composition Green Paint Sample	% Composition Blue Paint Sample
Hydroxyproline	7.95	6.41
Serine	3.29	3.36
Aspartic Acid	5.00	5.42
Glutamic Acid	6.36	5.50
Arginine/Threonine	5.57	4.92
Glycine	31.84	25.97
Alanine	10.80	8.97
Proline	9.97	8.19
Valine	0.59	0.67
Phenylalanine	1.89	1.70
Leucine/Isoleucine	-	3.85

Table 4.14: *Relative Percentage Amino Acid Composition*

The Body of Christ Borne to the Tomb (N01164)

Protein analysis of the green and blue paint samples revealed an amino acid composition consistent with that of animal glue in both cases. The sugar composition of the green paint was determined by GC-MS: the sample was found to be a mixed gum medium, most likely containing gum arabic, gum tragacanth and unrefined cane sugar, indicated by the presence of a significant amount of glucose in the sample. The priming appears to be predominantly karaya gum with added cane sugar, though the noticeable presence of xylose and fucose is perplexing in the obvious absence of gum tragacanth.

Sugar Component	Green Paint	Priming Sample
Arabinose	✓✓✓✓	
Rhamnose	✓✓✓	✓✓✓✓
Galactose	✓✓✓	✓✓✓
Mannose		
Fucose	✓	✓
Xylose	✓	✓✓
Glucose	✓✓✓✓	✓✓
Galacturonic Acid	✓	✓✓✓
Glucuronic Acid	✓	
Unknown	✓✓	✓✓

Table 4.15: *Monosaccharide Composition of Suspected Gum Media*
The Body of Christ Borne to the Tomb (N01164)

A second painting, *The Flight into Egypt* (Tate Gallery L01778, c. 1799), is also described as tempera on canvas. Again the work has undergone a moderate amount of retouching/consolidation with gelatine, though this should not have affected the samples, exhibiting an extensive amount of flaking/cracking. The work has also been varnished previously, the varnish layer having yellowed substantially over time. Samples of priming with pink

paint and brown paint with varnish residue were removed for protein analysis. It was observed that the actual paint layer was thin, allowing only limited sampling. Table 4.16 shows the relative percentage amino acid composition of the priming and paint samples.

The amino acid composition of both samples is consistent with that of animal glue. This work has a very thin paint layer, so each tiny sample contained less binding medium than usual – this lead to significant baseline noise, preventing accurate peak integration. The baseline was subsequently redrawn in both cases and peak areas were reintegrated.

Amino Acid Residue	% Composition Priming & Pink Paint Sample	% Composition Brown Paint & Varnish Residue Sample
Hydroxyproline	5.52	6.18
Serine	6.13	5.82
Aspartic Acid	10.43	10.91
Glutamic Acid	9.20	9.09
Arginine/Threonin	3.99	3.64
Glycine	25.15	24.00
Alanine	10.74	11.64
Proline	11.35	9.82
Valine	-	3.64
Phenylalanine	6.13	-
Tryptophan	4.60	5.45
Lysine	6.75	9.82

Table 4.16: *Relative Percentage Amino Acid Composition*
The Flight into Egypt (L01778)

Christ the Mediator (Tate Gallery L01779, c. 1799-1800) is comparable with the previous work. Described as tempera on canvas, the work has been consolidated with gelatine and displays extensive flaking/cracking of the paint surface. The previously applied varnish has

yellowed significantly, whilst a thin paint layer facilitated only limited sampling. A sample of dark paint with varnish residue was removed for protein analysis: table 4.17 shows the amino acid composition (relative percentages) of the sample.

The amino acid composition of the sample clearly indicates the presence of animal glue in the paint medium: a significant level of hydroxyproline, approximately 30 % glycine and an almost 1:1 ratio of alanine to proline are indicative of a collagen-based medium. Although it is known that Blake himself made extensive use of glue in his paint media, it is possible that the gelatine consolidation has also penetrated the paint layers.

Amino Acid Residue	% Composition Dark Paint Sample
Hydroxyproline	7.52
Serine	4.33
Aspartic Acid	5.19
Glutamic Acid	7.91
Arginine/Threonine	7.57
Glycine	30.77
Alanine	11.70
Proline	10.57
Valine	4.24
Phenylalanine	0.89
Leucine/Isoleucine	5.27
Lysine	4.03

Table 4.17: *Relative Percentage Amino Acid Composition*

Christ the Mediator (L01779)

Another tempera on canvas painting, *Bathsheba at the Bath* (Tate Gallery N03007, c. 1799-1800) [plate 2, appendix 3], has undergone considerable consolidation with gelatine, currently unexamined, and exhibits a reasonable amount of flaking/cracking of the paint surface. Samples of

priming with some paint and varnish and white/blue paint with varnish residue were removed and stored prior to protein/gum analysis. Tables 4.18 and 4.19 show the amino acid and sugar compositions of the samples.

The amino acid composition of the two samples is consistent with that of animal glue, though the sample of paint contained only a trace of glue – this seems to indicate that penetration of paint layers by gelatine used in consolidation is minimal. Since the amount of glue present in the paint layer is extremely small, some of the amino acid components were not detected, hence the slightly higher relative percentages of those observed (especially proline). The presence of very different gum media in the two samples was revealed by gum analysis *via* GC-MS. The priming layer was found to contain a mixed gum medium, in addition to animal glue, probably consisting of gums arabic and tragacanth with added cane sugar. However, the paint sample was found to contain mainly cane sugar, indicated by the presence of a very large amount of glucose, with traces of gums arabic and tragacanth.

Amino Acid Residue	% Composition Priming Sample	% Composition White/Blue Paint Sample
Hydroxyproline	5.27	9.15
Serine	3.47	13.41 in total
Aspartic Acid	6.02	
Glutamic Acid	5.67	3.66
Arginine/Threonine	4.44	3.66
Glycine	22.55	25.61
Alanine	8.72	8.54
Proline	7.62	9.51
Phenylalanine	2.81	4.88
Leucine/Isoleucine	4.79	9.76

Table 4.18: *Relative Percentage Amino Acid Composition Bathsheba at the Bath (N03007)*

Sugar Component	Priming Sample	Paint Sample
Arabinose	✓✓✓✓	✓
Rhamnose	✓✓✓	✓
Galactose	✓✓✓✓	✓
Mannose		
Fucose	✓✓	
Xylose	✓✓	✓
Glucose	✓✓✓	✓✓✓✓
Galacturonic Acid	✓	✓
Glucuronic Acid	✓	
Unknown	✓✓	✓✓✓

Table 4.19: *Monosaccharide Composition of Suspected Gum Media Bathsheba at the Bath (N03007)*

The Ghost of a Flea (Tate Gallery N05889, c. 1819-20) [plate 3, appendix 3] is believed to be tempera on a wooden panel. This work has not been consolidated, though microscopical examination shows an abundance of discoloured varnish, below which the paint is cracked extensively – indeed the crack pattern seems to suggest the presence of animal glue in either the paint or the priming. Samples of white priming (possibly commercial oil priming with adsorbed paint medium from its appearance in cross section), dark background paint (lower left corner) and blue paint (left edge) were removed for protein/gum analysis. Tables 4.20 and 4.21 show the amino acid and sugar compositions of the samples analysed.

Protein analysis revealed the presence of animal glue in all three samples, although the levels of glycine observed in the paint samples were slightly lower than expected. However, significant amounts of hydroxylproline, alanine and proline all confirm the presence of animal glue.

Amino Acid Residue	% Composition White Priming	% Composition Dark Background Paint	% Composition Blue Paint
Hydroxyproline	7.54	9.19	7.94
Serine	4.89	3.04	4.90
Aspartic Acid	9.16	6.10	10.86
Glutamic Acid	8.72	8.46	9.80
Arginine/ Threonine	5.08	4.40	6.59
Glycine	30.35	15.58	20.10
Alanine	14.05	20.90	20.73
Proline	9.75	16.62	7.61
Valine	2.17	-	3.02
Phenylalanine	0.28	7.33	2.94
Tryptophan	1.73	-	1.56
Leucine/Isoleucine	-	-	-
Lysine	6.27	8.37	1.76

Table 4.20: *Relative Percentage Amino Acid Composition*

The Ghost of a Flea (N05889)

Sugar Component	% Composition White Priming	% Composition Dark Background Paint	% Composition Blue Paint
Arabinose	√√√	√√√	√√√
Rhamnose	√√√	√√√√	√√√√
Galactose	√√√√	√√√	√√√
Mannose			
Fucose		√√	√√
Xylose		√√	√√
Glucose	√	√√√	√√√
Galacturonic Acid		√√	√√
Glucuronic Acid	√√	√√	√√
Unknown	√	√√	√√

Table 4.21: *Monosaccharide Composition of Suspected Gum Media*

The Ghost of a Flea (N05889)

Gum analysis by GC-MS seems to suggest that whilst the paint layers both contain mixed gum media, consisting of gums arabic, karaya (indicated by the large rhamnose component) and tragacanth (confirmed by the presence

of xylose and fucose) with added cane sugar, the priming layer contains only gum arabic.

The Agony in the Garden (Tate Gallery N05894, c. 1799-1800) [plate 4, appendix 3] is described as a tempera work on a steel support. The work has undergone a moderate amount of consolidation with gelatine, though the edges appear to have been consolidated with a paraffin wax. Extensive cracking of the paint surface is concealed by a layer of discoloured varnish. Samples of white priming and blue paint with a small amount of white priming (lower edge) were removed and submitted for protein/gum analysis. Tables 4.22 and 4.23 show the amino acid and sugar compositions of the samples.

Amino Acid Residue	% Composition White Priming	% Composition Blue Paint & Priming
Hydroxyproline	6.03	9.36
Serine	2.74	4.33
Aspartic Acid	4.70	5.56
Glutamic Acid	6.51	8.92
Arginine/Threonine	7.89	5.19
Glycine	23.11	21.67
Alanine	9.05	13.73
Proline	9.27	15.26
Valine	1.82	-
Phenylalanine	4.11	4.86
Leucine/Isoleucine	9.38	4.74
Lysine	9.65	4.56

Table 4.22: *Relative Percentage Amino Acid Composition*
The Agony in the Garden (N05894)

Again, both the priming and blue paint contain animal glue – the amino acid composition compares well with that seen for standard glue media. Results obtained for the GC-MS analysis of the paint/priming sample

also suggest the use of a mixed gum medium, probably containing mainly gum karaya with a little gum tragacanth and added cane sugar. However, the sample contains only a small amount of gum material, making identification more difficult.

Sugar Component	Blue Paint & Priming
Arabinose	√
Rhamnose	√√
Galactose	√√
Mannose	
Fucose	√
Xylose	√
Glucose	√√
Galacturonic Acid	√
Glucuronic Acid	√
Unknown	√√√

Table 4.23: *Monosaccharide Composition of Suspected Gum Media*
The Agony in the Garden (N05894)

Samples from two other tempera on canvas works, *The Spiritual Form of Nelson Guiding Leviathan* (Tate Gallery N03006, c. 1805-9) [plate 5, appendix 3] and *The Bard, from Gray* (Tate Gallery N03551, c. 1809) [plate 6, appendix 3], were submitted for gum analysis only. Both works have been consolidated with gelatine, though the nature of the consolidation remains unexamined, whilst the works display extensive and reasonable amounts of flaking/cracking respectively. Samples of white priming (possibly commercial oil priming with some adsorbed paint medium from its appearance in cross section) and paint medium with varnish residue were removed from *The Spiritual Form...* and two samples of clear, glossy paint

medium were taken from *The Bard, from Gray*. Table 4.24 shows the sugar composition of samples from both works.

Sugar Component	N03006 White Priming	N03006 Brown Paint Medium	N03551 S1 Clear Glossy Paint Medium	N03551 S2 Clear Glossy Paint Medium
Arabinose	√√	√√		√√√
Rhamnose	√√√	√√√√	√√√√	√√√√
Galactose	√√	√√	√√	√√
Mannose				
Fucose	√	√		√
Xylose	√√	√√√	√√	√√√
Glucose	√√√√	√√√	√√√	√√√
Galacturonic Acid	√	√	√	√
Glucuronic Acid	√	√	√	√
Unknown	√√	√√	√√	√√√

Table 4.24: *Monosaccharide Composition of Suspected Gum Media The Spiritual Form of Nelson Guiding Leviathan (N03006) and The Bard from Gray (N03551)*

The Spiritual Form of Nelson Guiding Leviathan appears to contain the same mixed gum medium in both the priming and brown paint samples. The most likely components of this mixture are karaya gum (since the rhamnose levels are disproportionately high compared with those seen for the other sugars), gum tragacanth (since both xylose and fucose are present) and cane sugar (indicated by high levels of glucose).

Samples from *The Bard, from Gray* also appear to contain mixed gum media. Sample S1 (clear glossy medium) contains no arabinose or mannose, which is indicative of karaya gum. However, the presence of xylose is anomalous in the absence of arabinose. A significant glucose component also

confirms the use of cane sugar. Sample S2, again described as clear, glossy medium, contains arabinose and both xylose and fucose, suggesting the presence of gum tragacanth. The large amounts of rhamnose observed indicate gum karaya and the use of cane sugar is indicated by the presence of significant amounts of glucose.

Finally, two watercolour works on gelatine-sized paper were studied: *The Blasphemer* (Tate Gallery N05195, c. 1800) [plate 7, appendix 3] and *The Simoniac Pope* (Tate Gallery N03357, c. 1824-27) [plate 8, appendix 3] are both believed to contain natural gum media. The works have not been retouched/consolidated in any way and neither has suffered any flaking or cracking. Samples of a thick, dark grey wash were removed from the lower edge of both paintings and submitted for gum analysis only. Upon analysis it was discovered that the sample from *The Blasphemer* had suffered contamination – the chromatogram showed the classic form of hydrocarbon contamination, so it was assumed that the septum used was not the recommended type for these samples, thus contamination had occurred. The sugar composition of the sample taken from *The Simoniac Pope* is shown in table 4.25.

The sample of dark grey wash appears to be a mixed gum medium probably containing cherry gum, gum tragacanth and karaya gum. A very large amount of xylose with a far smaller amount of fucose suggests that xylose is a component of more than one gum in the mixture, which leads to the conclusion that both cherry gum and gum tragacanth are present. Since neither of these gums contain significant levels of rhamnose and the arabinose

level is lower than that of rhamnose in the mixture, it is likely that gum karaya is the third gum present in the medium.

Sugar Component	N03357 Thick Dark Grey Wash
Arabinose	√√
Rhamnose	√√√
Galactose	√√
Mannose	
Fucose	√
Xylose	√√√
Glucose	
Galacturonic Acid	√
Glucuronic Acid	√
Unknown	√√

Table 4.25: *Monosaccharide Composition of Suspected Gum Media The Simoniac Pope (N03557)*

4.3.2 Works of Joseph Mallord William Turner (1775-1851)

A variety of paintings by J.M.W. Turner were studied, including watercolours and those suspected of containing proteinaceous binding media.

A sample of priming, possibly including some oil-based pale blue paint, was removed from *The Battle of Trafalgar, as Seen from the Mizen Starboard Shrouds of the Victory* (Tate Gallery N00480, c. 1806-8) [plate 9, appendix 3]. The work is on a canvas support, has previously been lined using animal glue and exhibits no flaking/cracking of the paint surface. Earlier GC analyses of other Turner works of this type have indicated the presence of an egg medium, thus the priming sample was submitted for protein analysis. The relative amino acid composition of the priming sample is shown in table 4.26.

When the relative amino acid composition of the sample was compared with that of the standard proteinaceous media it was clear that, despite the Tate's suspicion of an egg medium, the priming sample was in fact an animal glue.

Amino Acid Residue	% Composition Priming Sample
Hydroxyproline	9.14
Serine	2.84
Aspartic Acid	7.47
Glutamic Acid	7.18
Arginine/Threonine	5.90
Glycine	30.01
Alanine	9.75
Proline	16.14
Valine	3.91
Phenylalanine	3.44
Lysine	4.21

Table 4.26: *Relative Percentage Amino Acid Composition*
The Battle of Trafalgar... (N00480)

Turner used a hardwood panel as a drawing board for his painting *George IV's Departure from the 'Royal George'* (Tate Gallery N02880, c. 1822) [plate 10, appendix 3], possibly using some sort of animal glue to affix several layers of paper to the board. No consolidation/restoration work has been performed and the once transparent glue material has discoloured with age. Samples of this strong adhesive were taken and submitted for protein analysis. Table 4.27 shows the relative amino acid composition of the adhesive.

It was assumed that there was no animal glue present in the sample since the amount of hydroxyproline observed was very small (<2 %). The

very high percentage of glutamic acid indicates the presence of casein, well known as a particularly strong adhesive. However, high levels of both aspartic and glutamic acids usually suggest an egg medium, so it was proposed that the adhesive was a mixture of mainly casein with some added egg albumin. In order to confirm the nature of this apparently mixed medium, the experimental results obtained for the analyses of proteinaceous media, both known and unknown, were subjected to methods of statistical analysis and pattern recognition, *i.e.* cluster analysis, principal component analysis and discriminant function analysis. The results obtained from these statistical analyses can be found in chapter 5, section 5.4.2.

Amino Acid Residue	% Composition Adhesive Sample
Hydroxyproline	1.69
Serine	4.45
Aspartic Acid	10.15
Glutamic Acid	32.05
Arginine/Threonine	3.47
Glycine	8.93
Alanine	6.96
Proline	14.23
Valine	1.06
Phenylalanine	4.59
Leucine/Isoleucine	5.22
Lysine	3.92

Table 4.27: *Relative Percentage Amino Acid Composition*

George IV's Departure from the 'Royal George' (N02880)

Judith with the Head of Holofernes (Tate Gallery N05500, c. 1830) [plate 11, appendix 3], described as oil on canvas, has undergone no consolidation/restoration work and suffers a moderate amount of flaking/cracking. A sample of priming from the reverse was taken: a staining

test revealed the presence of proteinaceous material in the sample, which was subsequently submitted for protein analysis. The relative amino acid composition of the priming is shown in table 4.28.

The priming contained high levels of both aspartic and glutamic acids, indicating the presence of an egg medium. The level of glutamic acid is approximately double that expected for egg albumin, which could indicate the addition of casein to the medium. The level of proline is also higher than expected for a purely egg medium, again indicating the presence of casein. The very low level of hydroxyproline suggests that there is no animal glue in the medium, so it was concluded that the priming contained a mixed egg albumin and casein medium -- this was confirmed *via* statistical methods of pattern recognition and the results are shown in chapter 5, section 5.4.2.

Amino Acid Residue	% Composition Priming Sample
Hydroxyproline	2.09
Serine	4.67
Aspartic Acid	11.15
Glutamic Acid	25.82
Arginine/Threonine	4.21
Glycine	12.49
Alanine	10.54
Proline	8.68
Valine	3.10
Phenylalanine	7.54
Leucine/Isoleucine	3.60
Lysine	1.22

Table 4.28: *Relative Percentage Amino Acid Composition Judith with the Head of Holofernes (N05500)*

The Bridge and Goats (Tate Gallery D08147, c. 1806-7) [plate 12, appendix 3] is a watercolour on glue-sized paper from Turner's drawing

book, the *Liber Studiorum*. There is no evidence of any consolidation adhesive/treatment and the work displays a small amount of flaking/cracking. Fourier tranform infrared spectroscopy (FTIR) indicates the presence of gum and drying oil in the paint medium. A sample of the paint medium with burnt umber was submitted for gum analysis and the sugar composition obtained *via* GC-MS analysis is shown in table 4.29.

Sugar Component	Paint Medium with Burnt Umber
Arabinose	✓✓
Rhamnose	✓✓✓✓
Galactose	✓✓
Mannose	
Fucose	✓
Xylose	✓✓
Glucose	✓
Galacturonic Acid	✓
Glucuronic Acid	✓
Unknown	✓✓✓✓

Table 4.29: *Monosaccharide Composition of Suspected Gum Media*
The Bridge and Goats D08147

The paint medium was found to be a mixed gum medium, probably containing gums tragacanth and karaya. The presence of xylose and fucose with arabinose indicates gum tragacanth, whilst a disproportionately large rhamnose component suggests the use of karaya gum. Burnt umber is a mineral pigment and therefore does not contain any monosaccharide groups, which would contribute to the sugar profile, as part of its structure.

Two further watercolours were sampled for gum analysis: *Mont Pilatus from Lake Lucerne* (Tate Gallery D33496, c. 1845) [plate 13,

appendix 3] and *Lake of Lucerne, looking from Kussnacht towards the Bernese Alps; Mont Pilatus on the Right, Dark against the Sunset* (Tate Gallery D33499, c. 1845) [plate 14, appendix 3] are both watercolour and gouache on gelatine sized paper. Neither work is damaged, but both have been remounted at least once, probably using a glue adhesive, though such details are no longer available. A sample of fibres and the stain from the gouache was removed from each work and a sample of gouache itself was also removed from *Lake of Lucerne*..... Table 4.30 shows the sugar composition of samples removed from both works.

Sugar Component	D33496	D33499	
	Fibres & Stain from Gouache	Fibres & Stain from Gouache	Gouache
Arabinose	√√	√	√
Rhamnose	√√√√	√√√	√√√
Galactose	√√√	√√	√√
Mannose			
Fucose	√√	√√	√√
Xylose	√√	√	√
Glucose	√√√	√√√	√√√
Galacturonic acid	√√	√	√
Glucuronic Acid	√	√	√
Unknown	√√	√√√√	√√√√

Table 4.30: *Sugar Composition of Suspected Gum Media Mont Pilatus....(D33496) and Lake of Lucerne....(D33499)*

The composition of these samples closely resembles that of the sample of paint medium removed from Ward's *Sketch for Gordale Scar* (Tate Gallery N03703) [see section 4.3.4, page 99]. The presence of both xylose and fucose indicates the use of gum tragacanth, while a large amount of

rhamnose could be due to karaya gum. In fact, a number of unidentified peaks (retention times 12.59 and 15.44 minutes) found only in the karaya gum standards were observed, reinforcing the suggestion that karaya gum was also present in the paint medium. The glucose component was most likely from the paper fibres in the sample. It was concluded, therefore, that the paint medium for each of these works was a mixture of both gum tragacanth and karaya gum.

4.3.3 Works of Dante Gabriel Rossetti (1828-82)

Rossetti favoured multi-layer supports and he affixed backing paper to *The Tune of the Seven Towers* (Tate Gallery N03059, c. 1857) [plate 15, appendix 3] with a glue or gum adhesive. The work has undergone no consolidation and the backing paper is still adhering well. A sample of the adhesive was taken and submitted for protein analysis. Table 4.31 shows the relative amino acid composition of the adhesive.

Amino Acid Residue	% Composition Adhesive
Hydroxyproline	6.07
Serine	9.39
Aspartic Acid	19.05
Glutamic Acid	12.03
Arginine/Threonine	5.24
Glycine	29.54
Alanine	11.95
Proline	6.74

Table 4.31: *Relative Percentage Amino Acid Composition*

The Tune of the Seven Towers (N03059)

Baseline noise meant that the later eluting components, *i.e.* valine, phenylalanine, leucine, isoleucine and lysine, were partially obscured – these components were not included in the calculation of relative percentage composition. It is likely, therefore, that the relative percentages shown above are slightly higher than in reality, though it must be stressed that the differences will be small since the peak areas for all the major components were included in the calculation.

Despite this, the results clearly show that the adhesive sample contained animal glue – a significant level of hydroxyproline and around 30 % glycine confirm this. However, the levels of aspartic acid and glutamic acid are higher than usual for an animal glue, bearing more resemblance to those seen in egg media. A mixed animal glue and egg adhesive was suspected, thus the results were interpreted using statistical methods of pattern recognition, the outcome of which confirmed the nature of the mixed adhesive. Details of the statistical analyses used are shown in chapter 5, section 5.4.2.

Dr. Johnson at the Mitre (Tate Gallery N03827, c. 1860) [plate 16, appendix 3] and *How Sir Galahad, Sir Bors and Sir Percival were Fed with the Sanct Grael, but Sir Percival's Sister Died by the Way* (Tate Gallery N05234, c. 1864) [plate 17, appendix 3] are both painted on glue-sized paper and a gum medium was suspected in both works. Neither painting has undergone any form of consolidation or restoration treatment. *Dr. Johnson at the Mitre* exhibits a small amount of flaking/cracking and there has been a slight loss of colour from the crimson organic pigments. The paint is very medium-rich and glossy, blooming in patches: a sample of paint medium was

removed from a bloomed area and submitted for gum analysis. *How Sir Galahad...* exhibits a moderate amount of flaking/cracking, but some areas of the work have extensive cracking which is not suggestive of a gum medium. There is no evidence of any loss of colour from the pigments used. A sample of red paint with a suspected gum or egg medium was removed for gum analysis – there was insufficient sample for protein analysis as well.

Table 4.32 shows the sugar compositions obtained *via* GC-MS analysis for the paint media samples taken from these two paintings.

Sugar Component	Paint Medium N03827	Paint Medium N05234
Arabinose		
Rhamnose		
Galactose	✓✓	✓✓
Mannose		
Fucose	✓✓✓✓	
Xylose	✓✓	✓✓✓
Glucose		
Galacturonic Acid		
Glucuronic Acid		
Unknown	✓✓✓✓	✓✓✓✓

Table 4.32: *Monosaccharide Composition of Suspected Gum Media Dr. Johnson at the Mitre (N03827) and How Sir Galahad...(N05234)*

The results obtained for both these samples did not compare with those seen for any of the standard gum media samples analysed. It has been reported that tamarind seed mucilage contains xylose and galactose,[20] with very few other components, so it is possible that the paint medium taken from *Dr. Johnson at the Mitre* contains tamarind seed mucilage: however, this

would be considered very unusual as its use is thought to be primarily confined to Asia.

Since an egg or gum medium was suspected for the sample from *How Sir Galahad*.... the sugar composition of egg was ascertained – the results obtained did not confirm the identity of the paint medium. Galactose is the primary structural monosaccharide of many carbohydrate compounds, but in brown seaweeds the main sugar building unit is fucose, forming the seaweed polysaccharide fucoidin.[114] It is known that mucilages obtained from seaweeds have been used in Asia and the Far East, though their use elsewhere is not commonplace. However, fucose is clearly the major sugar component in the sample of paint medium, hence it is possible that an unusual seaweed mucilage has been used. The work has areas of extensive cracking that does not usually occur with a gum medium, but the use of a seaweed gum with a different carbohydrate structure may account for this.

4.3.4 Other Works

A number of works from the Tudor period (1485-1603) were investigated. Hans Holbein (1497-1543), a native German living in London, became a favourite of Henry VIII and his style inspired many followers: indeed *William, First Lord de la Warr* (Tate Gallery N04252, c. 1550) is attributed to one of his followers. The work is described as oil on a hardwood (possibly oak) panel, has undergone no consolidation/restoration and displays only a small amount of flaking/cracking. A sample of priming was removed for protein analysis, since it is possible that the priming from a work of this

period could contain animal glue. The relative percentage amino acid composition of the priming sample is shown in table 4.33.

The detector range was adjusted to increase its sensitivity, which lead to an increase in baseline interference that masked the later eluting peaks. However, the amino acid composition shows the presence of animal glue (approximately 25 % glycine and a significant amount of hydroxyproline), though the elevated levels of aspartic and glutamic acids could be caused by the addition of casein to the mixture.

Amino Acid Residue	% Composition Priming Sample
Hydroxyproline	5.18
Serine	6.19
Aspartic Acid	12.09
Glutamic Acid	18.81
Arginine/Threonine	5.42
Glycine	28.84
Alanine	11.51
Proline	11.95

Table 4.33: *Relative Percentage Amino Acid Composition*

William, First Lord de la Warr (N04252)

Egg proteins contain higher levels of serine and, as the level of serine in the sample is not unusually high, the addition of egg can be excluded. Statistical methods of pattern recognition were used to confirm the nature of the mixed medium – results of these analyses can be found in chapter 5, section 5.4.2.

An Allegory of Life (Tate Gallery T05729, c. 1570), from the British School, is also described as oil on an oak panel. The panel may have been glue-sized before priming, whilst a little surface consolidation and filling has

been performed. The small amount of surface cracking has a pattern suggestive of glue-based priming.

The upper layer of priming was previously found to be lead white in oil. A sample of the lower priming layer, likely to be glue-based, was removed for protein analysis - the relative percentage amino acid composition is shown in table 4.34.

Amino Acid Residue	% Composition Priming Sample
Hydroxyproline	8.09
Serine	3.60
Aspartic Acid	7.30
Glutamic Acid	6.74
Arginine/Threonine	1.87
Glycine	27.27
Alanine	9.10
Proline	9.27
Valine	2.04
Phenylalanine	1.65
Leucine/Isoleucine	3.51

Table 4.34: *Relative Percentage Amino Acid Composition*
An Allegory of Life (T05729)

On analysis the priming was found to contain animal glue – the amino acid composition of the sample is comparable with that for standard animal glue media, containing approximately 30 % glycine, a significant level of hydroxyproline and an almost 1:1 ratio of alanine to proline.

Cornelis Ketel painted a series of family portraits in 1579-80, *Alice Smythe, Robert Smythe* and *Joan Smythe.* The works are described as oil on hardwood panels and are believed to have glue-based priming. None of the works have undergone any consolidation/restoration and all three display a

small amount of flaking/cracking of the paint surface. The varnish layer was removed prior to sampling. Samples of priming were removed for protein analysis. Table 4.35 shows the relative percentage amino acid composition of each of the priming samples.

Amino Acid	Alice Smythe Priming	Robert Smythe Priming	Joan Smythe Priming
Hydroxyproline	7.34	4.36	2.26
Serine	3.30	28.74	3.93
Aspartic Acid	12.71	in total	26.55
Glutamic Acid	7.94	4.25	4.05
Arginine/ Threonine	3.12	3.64	5.42
Glycine	33.54	26.41	25.67
Alanine	9.76	9.87	10.32
Proline	10.56	7.10	5.52
Valine	6.49	9.04	5.30
Phenylalanine	5.25	2.60	5.25

Table 4.35: *Relative Percentage Amino Acid Composition*
Alice Smythe, Robert Smythe and Joan Smythe

The level of glycine in each sample suggests the presence of animal glue in the priming, though the amount of hydroxyproline in the sample from *Joan Smythe* is rather lower than expected. The priming from *Alice Smythe* is probably animal glue with a little added egg: this would account for the levels of aspartic and glutamic acids which are a little higher than in animal glue alone. Methods of pattern recognition were used to confirm the nature of the mixed medium (see chapter 5, section 5.4.2).

The priming samples from both *Robert Smythe* and *Joan Smythe* are similar in that the aspartic acid levels are abnormally high, especially when the level of glutamic acid in each remains near normal for an animal glue

medium. This again seems to indicate the use of a mixed medium in the priming, hence the results were subjected to statistical methods of pattern recognition in the hope that the other component of the mixture could be identified. The use of a mixed animal glue and egg medium in the priming samples from both these works was suggested by discriminant function analysis: the results obtained *via* this analysis can be found in chapter 5, section 5.4.2.

Joshua Reynolds' work *Sir James Hodges* (Tate Gallery N03545, c. 1765), described as oil on canvas, has undergone extensive treatment with a wax/resin lining adhesive and exhibits a small amount of surface flaking/cracking. Reynolds (1723-92) used mixed media and the priming from this work may include animal glue: a sample of priming was submitted for protein analysis. Table 4.36 shows the relative percentage amino acid composition obtained by RP-HPLC analysis of the sample.

Amino Acid Residue	% Composition Priming Sample
Hydroxyproline	5.79
Serine	7.72
Aspartic Acid	9.47
Glutamic Acid	15.47
Arginine/Threonine	4.96/4.11
Glycine	21.59
Alanine	8.42
Proline	11.08
Valine	2.43
Leucine/Isoleucine	10.23
Lysine	3.03

Table 4.36: *Relative Percentage Amino Acid Composition Sir James Hodges (N03545)*

The significant amounts of hydroxyproline and glycine, plus the almost 1:1 ratio of alanine to proline, indicate the presence of animal glue, whilst the high levels of glutamic and aspartic acids and serine are more usually seen in egg protein. A mixed egg and glue medium was suspected and the results were subjected to statistical methods of pattern recognition in order to confirm the exact nature of the medium in the priming sample: the results obtained from discriminant function analysis, which also suggest the presence of casein in the mixture, can be seen in chapter 5, section 5.4.2.

Edward Matthew Ward (1816-79) often produced sketches for his paintings and *Sketch for Gordale Scar* (Tate Gallery N03703, c. 1811) is an unvarnished watercolour and mixed media work on paper. A small amount of retouching/consolidation has been performed, though the work does not exhibit any flaking/cracking. A sample of paint medium from a transparent area of the painting was removed for analysis: the sugar composition of the sample is shown in table 4.37.

Sugar Component	Paint Medium from Transparent Area
Arabinose	✓✓
Rhamnose	✓✓✓✓✓
Galactose	✓✓
Mannose	
Fucose	✓
Xylose	✓✓
Glucose	
Galacturonic Acid	✓
Glucuronic Acid	
Unknown	✓✓✓

Table 4.37: *Monosaccharide Composition of Suspected Gum Medium Sketch for Gordale Scar (N03703)*

The appearance of both of xylose and fucose is indicative of gum tragacanth, though the table clearly shows the overwhelming presence of rhamnose in the sample. The level of rhamnose far exceeds that seen in karaya gum samples so investigations were made into the nature of the pigment used, since it has been reported that certain pigments contain monosaccharides as an integral part of their structure. Quercitin (a natural yellow pigment obtained from quercitron, the inner bark of the American oak *Quercus tinctoria*) is primarily found as the glucoside quercitrin, which has L-rhamnose condensed with the C3 hydroxyl group (see figure 4.7).[115] Confirmation that the sample contained a yellow pigment led to the conclusion that the paint medium was in fact gum tragacanth.

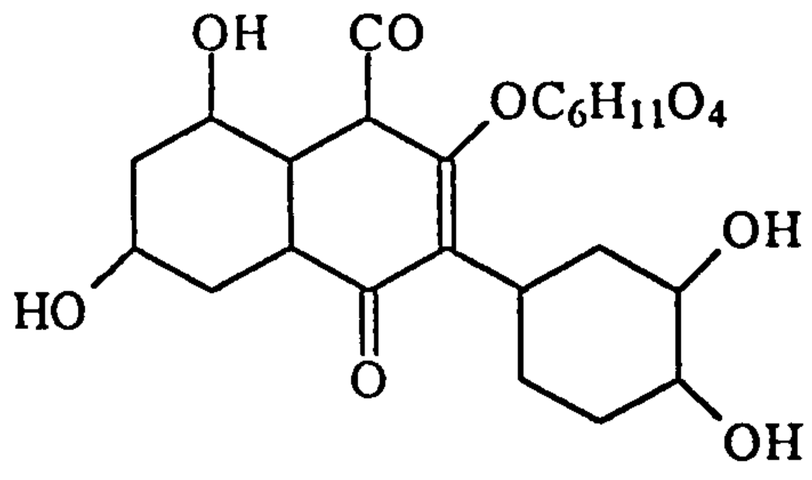


Figure 4.7: *Quercitrin · Precursor to Quercetin*

Samples from a selection of more modern works were also analysed. *The Ass* (Tate Gallery L01615, c. 1913), by Duncan Grant, is an oil painting on paper. A small amount of consolidation has been performed away from the area of sampling and the work suffers from a little flaking/cracking.

Areas of the work's priming supported a colony of wasps and it is known that Grant used aqueous media, *e.g.* casein. A sample of priming from this colonised area was therefore submitted for protein analysis: the relative amino acid composition of the sample is shown in table 4.38.

Amino Acid Residue	% Composition Priming Sample
Serine	2.86
Aspartic Acid	7.57
Glutamic Acid	29.90
Arginine/Threonine	2.39
Glycine	8.68
Alanine	5.48
Proline	11.49
Valine	7.05
Phenylalanine	4.27
Leucine/Isoleucine	7.84

Table 4.38: *Relative Percentage Amino Acid Composition The Ass (L01615)*

The composition of the priming sample was found to be consistent with that of casein, containing a large amount of glutamic acid (>15 %), greater than 8 % proline and around 7 % aspartic acid.

An egg tempera medium was suspected for Edward Wadsworth's work, *Near Marseilles* (Tate Gallery Wads2, c. 1922). A sample of paint, possibly with some priming, was taken from an unconsolidated area after varnish removal. Protein analysis was performed and table 4.39 shows the relative amino acid composition of the paint medium.

It appears that the paint medium contains egg protein. The levels of glutamic and aspartic acids and leucine are similar to those seen in standard egg white samples: this conclusion is reinforced by the presence of

approximately 3 % threonine and 5 % serine – egg yolk contains higher levels of both these amino acids. The small amount of hydroxyproline present seems to indicate that the sample does indeed contain some animal glue-based priming, which may account for the slightly elevated levels of proline and glycine.

Amino Acid Residue	% Composition Priming Sample
Hydroxyproline	4.06
Serine	4.69
Aspartic Acid	7.33
Glutamic Acid	9.55
Arginine/Threonine	2.11/2.88
Glycine	12.82
Alanine	5.98
Proline	19.21
Valine	4.15
Phenylalanine	3.57
Leucine	11.49
Lysine	2.93

Table 4.39: *Relative Percentage Amino Acid Composition Near Marseilles (Wads2)*

FTIR and staining tests suggest that Lessore's *Apollo and Daphne* (Tate Gallery T06693, c. 1985) has a mixed oil and protein paint medium, glue and egg both being possible components of the mixture. A sample of the titanium white priming was removed for protein analysis: table 4.40 shows the relative percentage amino acid composition of the priming.

The amino acid composition of the priming sample is consistent with that of animal glue, *i.e.* greater than 2 % hydroxyproline, a high level of glycine (approximately 25 %) and an almost 1:1 ratio of alanine to proline.

Amino Acid Residue	% Composition Priming Sample
Hydroxyproline	8.76
Serine	2.58
Aspartic Acid	5.63
Glutamic Acid	6.07
Arginine/Threonine	3.12
Glycine	23.74
Alanine	8.54
Proline	10.70
Valine	3.67
Lysine	4.23

Table 4.40: *Relative Percentage Amino Acid Composition Apollo and Daphne (T06693)*

Finally, samples of a coating removed from a sculpture were submitted for analysis. *The Rescue of Andromeda* (Tate Gallery N01749, c. 1895) by Fehr is a bronze sculpture with a wax-based coating, applied in the 1950s. The coating has degraded, is subsequently resistant to solvents and is not easily melted, even by a propane burner. Beeswax and lanolin are listed components of the coating, yet they melt easily – a possible proteinaceous composition was therefore suspected. Table 4.41 shows the relative percentage amino acid composition of the coating obtained *via* RP-HPLC analysis.

The chromatogram obtained for this analysis was particularly poor, probably due to non-proteinaceous contaminants in the sample, *e.g.* wax. There was a high level of baseline interference making integration difficult and obscuring most of the later eluting peaks. The relative percentage amino acid composition appears to indicate the presence of a mixed medium, possibly containing animal glue and egg protein. Hydroxyproline at greater than 2 % and approximately 30 % glycine confirm the presence of animal

glue, whilst high levels of both aspartic and glutamic acids usually indicate an egg-based medium.

Amino Acid Residue	% Composition Surface Coating Sample
Hydroxyproline	3.31
Serine	19.83 in total
Aspartic Acid	
Glutamic Acid	12.58
Arginine/Threonine	5.83
Glycine	27.22
Alanine	11.73
Proline	6.94

Table 4.41: *Relative Percentage Amino Acid Composition*

The Rescue of Andromeda (N01749)

Egg yolk has a combined total percentage of serine and aspartic acid of approximately 20 % (this figure is around 4 % lower in egg albumin), which is comparable with that seen in this sample. Casein is excluded as a component of the mixture since the glutamic acid level is too low, whilst the serine/aspartic acid level is far too high. The data was subjected to discriminant function analysis, in order to confirm the exact nature of the suspected animal glue-egg yolk medium: the results of the statistical analysis can be found in chapter 5, section 5.4.2.

Tables 4.42 to 4.44 summarise all the results obtained for samples of priming, paint media, watercolour media and adhesives removed from works from the Tate Gallery's collection, whilst selected chromatograms obtained from the analysis of samples of suspected proteinaceous and gum media removed from works of art can be found in appendix 4.

Artist/Title of Work	Support	Sample	Medium	Comments
BLAKE, c. 1799-1800 *Body of Christ Borne to the Tomb* (N01164)	Canvas	Green paint	Animal glue, gums arabic, tragacanth and cane sugar.	Reasonable amount of flaking/ cracking. Reasonable amount of consolidation but nature of work not examined.
		Blue paint from sky	Animal glue	
BLAKE, c. 1799 *The Flight into Egypt* (L01778)	Canvas	Pink paint and priming	Animal glue	Extensive flaking/cracking. Consolidation, probably with layer of animal glue.
		Brown paint	Animal glue	
BLAKE, c. 1799-1800 *Christ the Mediator* (L01779)	Canvas	Dark paint	Animal glue	Extensive flaking/cracking. Moderate consolidation with varnish or animal glue.
BLAKE, c. 1799-1800 *Bathsheba at the Bath* (N03007)	Canvas	White and blue paint	Animal glue with cane sugar and traces of gums arabic and tragacanth	Reasonable amount of flaking/ cracking. Reasonable level of consolidation but unexamined.
BLAKE, c. 1819-1820 *Ghost of a Flea* (N05889)	Wooden panel	Dark background paint	Animal glue, gums arabic, karaya, tragacanth and cane sugar	Extensive flaking/cracking. Crack pattern suggests animal glue in priming/ground or paint layers.
		Blue paint	Animal glue, gums arabic, karaya, tragacanth and cane sugar	
BLAKE, c. 1799-1800 *Agony in the Garden* (N05894)	Steel plate	Blue paint plus a little priming	Animal glue plus mainly gum karaya with a little gum tragacanth and cane sugar	Extensive flaking/cracking. Consolidation with varnish or animal glue.

Table 4.42: *Summary of Results Obtained from Analysis of Paint Media*

Artist/Title of Work	Support	Sample	Medium	Comments
BLAKE, c. 1805-9 *Spiritual Form of Nelson Guiding Leviathan* N03006	Canvas	Paint medium with varnish residue	Gums karaya, tragacanth and cane sugar	Extensive flaking/ cracking. Moderate amount of consolidation but nature of work not examined
BLAKE, c. 1809 *The Bard, from Gray* (N03551)	Canvas	S1 Clear glossy paint medium	Karaya gum and cane sugar	Reasonable amount of flaking/ cracking. Reasonable amount of consolidation but nature of work not examined
		S2 Clear glossy paint medium	Gums karaya, tragacanth and cane sugar	
BLAKE, c, 1824-27 *The Simoniac Pope* (N03357)	Gelatine-sized paper	Thick dark grey wash	Gums karaya, tragacanth and cherry	No flaking/cracking or consolidation. Sample is quite glossy.
ROSSETTI, c. 1860 *Dr. Johnson at the Mitre* (N03827)	Glue-sized paper	Paint medium from bloomed area	Possibly tamarind seed mucilage: result did not correspond with any standard gum samples.	A little flaking/cracking. No consolidation or restoration. Medium-rich paint, blooming in patches.
ROSSETTI, c. 1864 *How Sir Galahad....* (N05234)	Glue-sized paper	Red paint	Possibly brown seaweed mucilage: result did not correspond with any standard gum samples.	Overall, moderate flaking/ cracking but certain areas have extensive cracking not characteristic of gum medium.
WADSWORTH, c. 1922 *Near Marseilles* (Wads 2)	Canvas	Paint	Egg albumin and animal glue mixture	A little flaking/cracking.

Table 4.42: *Summary of Results Obtained from Analysis of Paint Media*

Artist/Title of Work	Support	Sample	Medium	Comments
BLAKE, c. 1799-1800 *Body of Christ Borne to the Tomb* (N01164)	Canvas	Priming	Karaya gum and cane sugar	Reasonable amount of flaking/ cracking. Reasonable amount of consolidation but nature of work not examined
BLAKE, c. 1799-1800 *Bathsheba at the Bath* (N03007)	Canvas	Priming	Animal glue with gums arabic and tragacanth, plus added cane sugar	Reasonable amount of flaking/ cracking. Reasonable amount of consolidation but nature of work not examined
BLAKE, c. 1819-1820 *Ghost of a Flea* (N05889)	Wooden panel	Priming	Animal glue and gum arabic mixture.	Extensive flaking/cracking. Crack pattern suggests animal glue in priming/ground or paint layers.
BLAKE, c. 1799-1800 *Agony in the Garden* (N05894)	Steel plate	Priming	Animal glue	Extensive flaking/cracking. Consolidation with varnish or animal glue.
BLAKE, c. 1805-9 *Spiritual Form of Nelson....* (N03006)	Canvas	White priming	Karaya gum with gum tragacanth and cane sugar	Extensive flaking/ cracking. Moderate amount of consolidation but nature of work not examined
TURNER, c. 1806-8 *The Battle of Trafalgar...* (N00480)	Canvas	Priming	Animal glue	No flaking/cracking. Extensive consolidation. Glue lining may have impreganted priming.
TURNER, c. 1830 *Judith with the Head of Holofernes* (N05500)	Canvas	Priming	Egg albumin and casein mixture	Moderate flaking/cracking. No consolidation/restoration.

Table 4.43: *Summary of Results Obtained from Analysis of Priming Samples*

Artist/Title of Work	Support	Sample	Medium	Comments
Follower of Holbein, c. 1550 *William, First Lord de la Warr* (N04252)	Hardwood panel	Priming	Animal glue and casein mixture	Small amount of flaking/ cracking. No consolidation or retouching.
British School, c. 1570 *An Allegory of Life* (T05729)	Oak panel	Priming	Animal glue	Small amount of consolidation and retouching. Panel may have been glue-sized before priming. Crack pattern suggests glue priming.
KETEL, c. 1579-80 *Alice Smythe, Robert Smythe* and *Joan Smythe*	Hardwood panels	Priming	Animal glue with added egg yolk	Small amount of flaking/ cracking. No consolidation or retouching.
REYNOLDS, c. 1765 *Sir James Hodges* (N03545)	Canvas	Priming	Animal glue, egg and casein mixture	Small amount of flaking/ cracking. Extensive consolidation.
GRANT, c. 1913 *The Ass* (L01615)	Paper	Priming	Casein	A little flaking/cracking. Some consolidation, but away from sampling site.
LESSORE, c. 1985 *Apollo and Daphne* (T06693)	Canvas	Priming	Animal glue	No flaking or cracking. No consolidation or retouching.

Table 4.43: *Summary of Results Obtained from Analysis of Priming Samples*

Artist/Title of Work	Support	Sample	Medium	Comments
BLAKE, c. 1824-27 *The Simoniac Pope* (N03357)	Gelatine-sized paper	Thick dark grey wash	Mixture of gums karaya, tragacanth and cherry	No flaking or cracking. No consolidation or retouching. Sample is quite glossy.
TURNER, c. 1806-7 *The Bridge and Goats* (D08147)	Glue-sized paper	Paint medium with burnt umber	Mixture of gums tragacanth and karaya	Small amount of flaking/cracking. No signs of consolidation adhesives or treatments to reverse.
TURNER, c. 1845 *Mont Pilatus....* (D33496)	Glue-sized paper	Stain from gouache and fibres	Mixture of gums tragacanth and karaya	No damages to work. Remounted at least once.
TURNER, c. 1845 *Lake of Lucerne....* (D33499)	Glue-sized paper	Stain from gouache/fibres Gouache	Mixture of gums tragacanth and karaya in both samples	No damages to work. Remounted at least once.
WARD, c. 1811 *Sketch for Gordale Scar* (N03703)	Paper	Paint medium from transparent area	Gum tragacanth	No flaking or cracking. Small amount of consolidation but should not affect analysis.
TURNER, c. 1822 *George IV's Departure....* (N02880)	Hardwood panel drawing board	Adhesive between paper and drawing board	Predominantly casein with a little added egg albumin	Paper still adhering well.
ROSSETTI, c. 1857 *Tune of the Seven Towers* (N03059)	Backing paper	Adhesive between backing paper and work	Animal glue and egg yolk mixture	Paper still adhering well. No consolidation.
FEHR, c. 1895 *The Rescue of Andromeda* (N01749)	Bronze	Wax-based coating applied in the 1950s	Animal glue and egg yolk in the coating	Small amount of flaking/cracking of coating. No consolidation.

Table 4.44: *Summary of Results Obtained from Analysis of Watercolour Media and Adhesive Samples*

CHAPTER 5

Chemometric Studies

5.1 Introduction to Pattern Recognition Techniques[116]

Where a study requires the analysis and interpretation of large volumes of samples and data, statistical methods of pattern recognition can be employed to help "sort" the data. There are two broad categories of pattern recognition procedures – ***unsupervised*** and ***supervised.***

Unsupervised techniques determine the relationship between the samples without any prior information about the samples. Unsupervised techniques include cluster analysis and principal component analysis (PCA).

Supervised techniques assume that the samples fall into specified groups or classes, each group being defined by a model. The group models are derived from a set of samples whose composition, thus group or class, is known. Thus, unknown samples are classified by calculation of the "distances" of the unknowns from the groups within the training set. This concept is diagramatically illustrated in figure 5.1.

Supervised techniques can be further subdivided into two models – hard modelling and soft modelling. In hard modelling techniques, a sample is directly placed into a specified group, even if this can only be achieved with difficulty: discriminant analysis is a widely used hard modelling technique. Soft modelling, a much newer concept, involves the assignment of a probability of membership of a sample to a predetermined group and may even require the placement of the sample in a completely new group or class.

Discriminant analysis (also known as discriminant function analysis) and principal component analysis techniques were employed here to assist in the interpretation of experimental results obtained from the analysis of samples removed from selected works of art where a mixed medium was suspected.

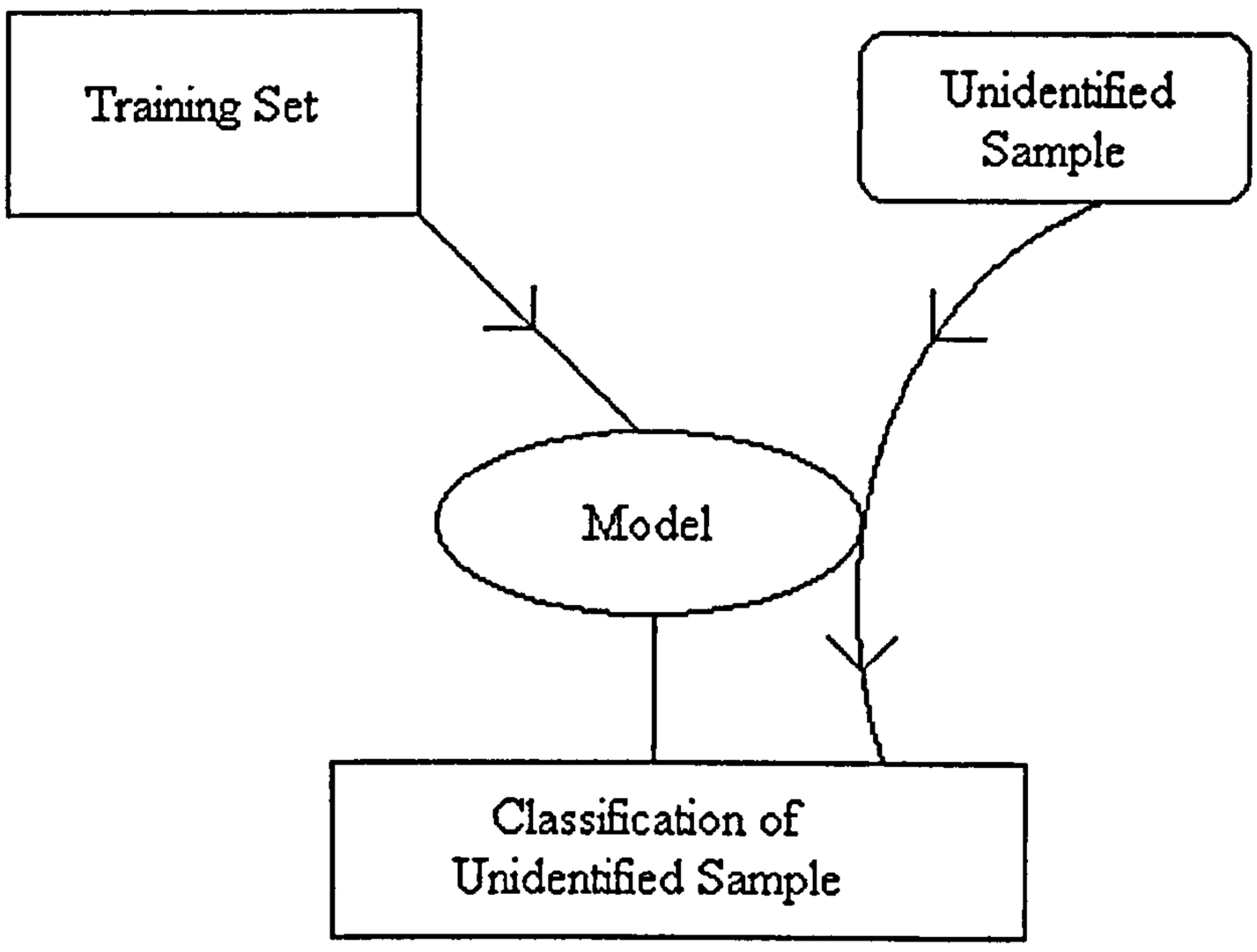


Figure 5.1: *Flow Diagram Illustrating Stages of Supervised Pattern Recognition*

5.2 Principal Component Analysis[116]

5.2.1 Introduction to PCA and its Applications

Principal component analysis (PCA) facilitates the simplification of large data sets by reducing the number of independent variables, detecting

structure in the relationship between variables and cases for classification purposes.

The technique is widely used to extract and interpret information from multivariate data. Consider a multivariate data matrix for N objects, with χ variables per object. PCA simplifies the matrix by data reduction using modelling, outlier detection, variable selection, classification and prediction.

The original variables are transformed to new axes (principal components) so that the data scores expressed on these axes are uncorrelated, *i.e.* the principal components are orthogonal (they can be determined uniquely). PCA allows the maximum amount of variation in the data matrix to be represented by the minimum number of principal components (PCs), thus each successively derived PC expresses a decreasing amount of the total variation.

Principal components are derived as follows. If two variables, V_1 and V_2, are plotted against each other a regression line describing the trend in the two variables can be drawn (figure 5.2): this regression line is the first principal component (PC_1) and is described by the equation $PC_1=AV_1 + BV_2$, where A and B are regression coefficients and are called factor loadings. In this particular case, the only remaining "trend" is the scatter repeated by a second PC (PC_2) at 90 ° to PC_1. The second principal component (PC_2) is equal to the scattering in the data and is orthogonal since it is at 90 ° to the PC_1 regression line. The two original variables are therefore replaced by PC_1, which represents a rotation of the original V_1/V_2 axes to show the greatest variation in the data – this is known as a "varimax" rotation (variance maximising rotation). After PC_1 has been extracted, it is possible to draw

further lines which maximise the remaining variability in the data: these consecutive factors are also orthogonal or uncorrelated. PCs may be usefully extracted until only a little random variability, (*e.g.* PC_2 in the above example), rather than a discernible trend remains in the data matrix.

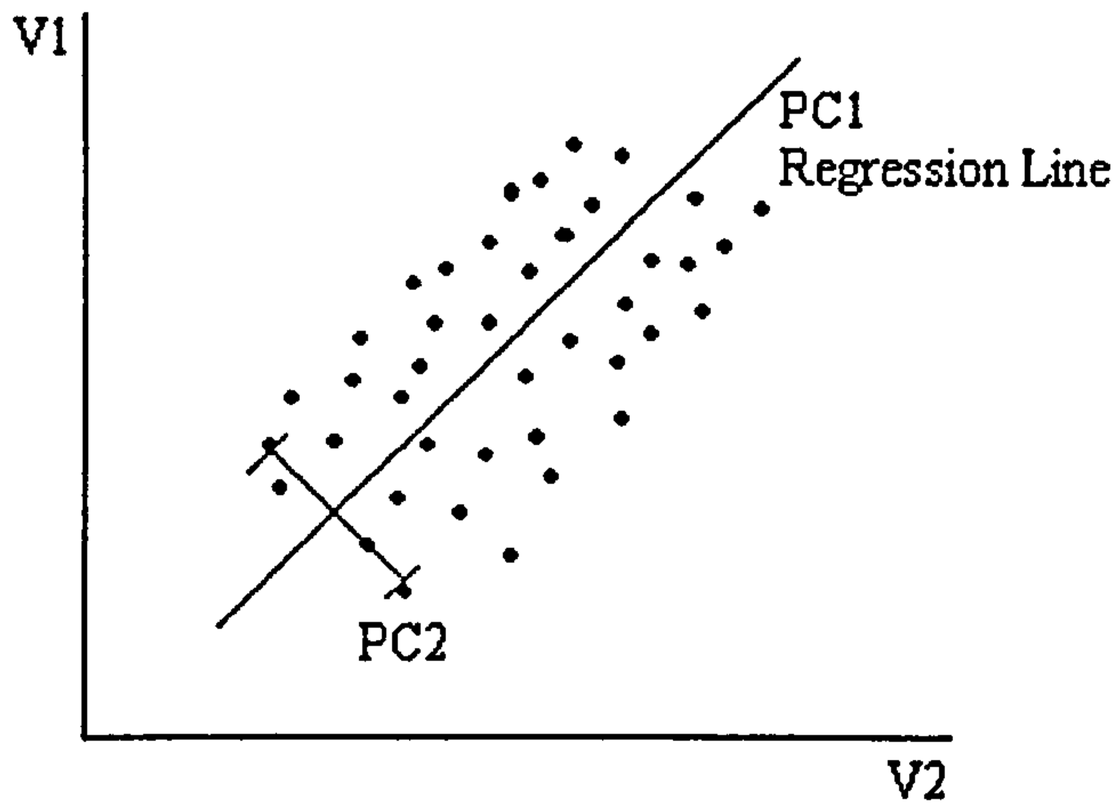


Figure 5.2: *Plot of Variables Showing Extraction of Principal Components*

The optimum number of extractable PCs can be ascertained by the use of "eigenvalues". The original variables are standardised to the same scale by subtraction of the mean and division by the standard deviation: all standardised variables then have a mean of zero and a standard deviation and variance of 1. Thus the total variance is equal to the number of variables. Now the total variance represented by a PC is called its "eigenvalue" and as successive PCs represent decreasing variance, so the eigenvalues decrease.

Scree plots and the Kaiser criterion are methods which utilise eigenvalues to ascertain the optimum number of PCs.

Scree plots identify those PCs which represent only the random variability in the data: such PCs are shown as the tail of the curve of the plot of eigenvalues against principal components (figure 5.3).

Figure 5.3: *An Example of a Scree Plot*

Kaiser stated that only those PCs with an eigenvalue greater than 1 should be extracted, since a lower eigenvalue indicates random variability.

5.2.2 Classification by PCA

Unknown samples can be classified using PCA by calculation of factor loadings or factor scores.

Consider the regression equation $PC_1=A(X_1) + B(X_2)$. If X_1 and X_2 are the standardised values of variables V_1 and V_2, then A and B are coefficients which describe the relative importance of each variable to PC_1, *i.e.* factor loadings. Scatter plots of factor loadings between two PCs can reveal useful information on the relationship of variables to the PCs: these relationships may also be defined by plotting the correlation against the PCs. The correlation can be determined using equation 5.1:

$$\text{Correlation} = \{\text{Factor loading}\} * \{\sqrt{\text{eigenvalue}}\} \qquad \textbf{Eq. 5.1}$$

The length of each vector on the correlation plot represents how much of the standardised variable has been described by a particular PC (figure 5.4). It can be seen that X_2 and X_4 are highly correlated with PC_2 and PC_1 respectively, whilst X_3 and X_6 have little importance to either PC.

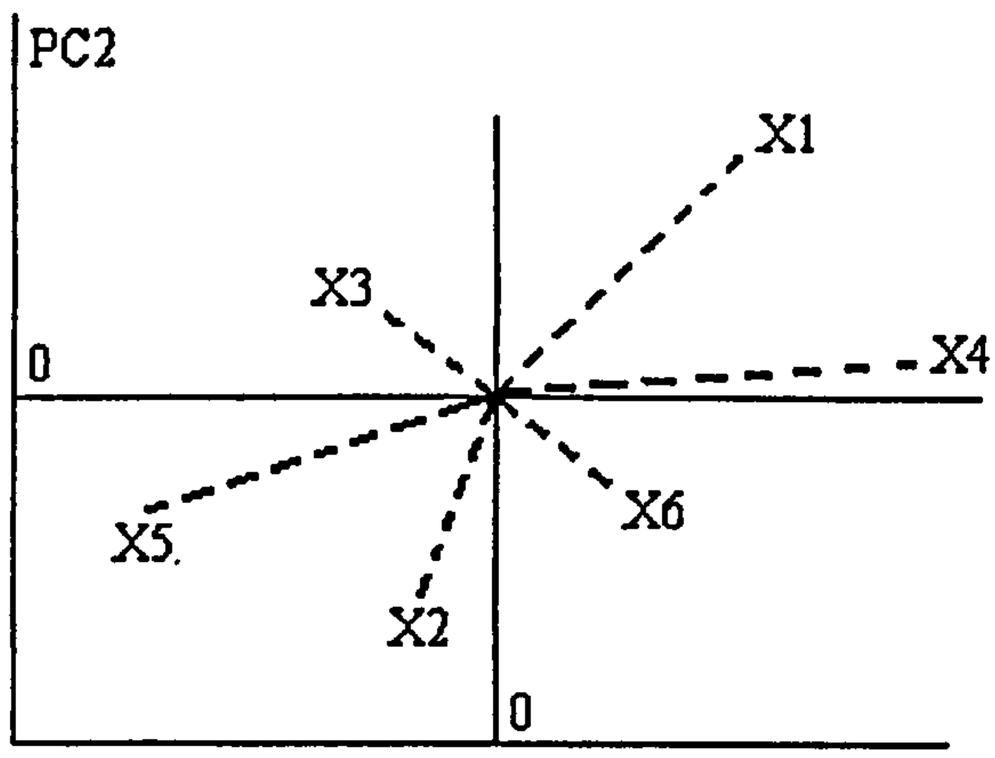


Figure 5.4: *Correlation Plot of Standardised Variables*

A factor score is the value of an individual sample for any particular PC, *i.e.* the numerical value of $A(X_1) + B(X_2)$, with values of X_1 and X_2 corresponding to a given sample. Factor scores are plotted in the same way as factor loadings (figure 5.5). Scatter plots of factor loadings and scores are complementary and can be used to determine which particular variables discriminate between samples.

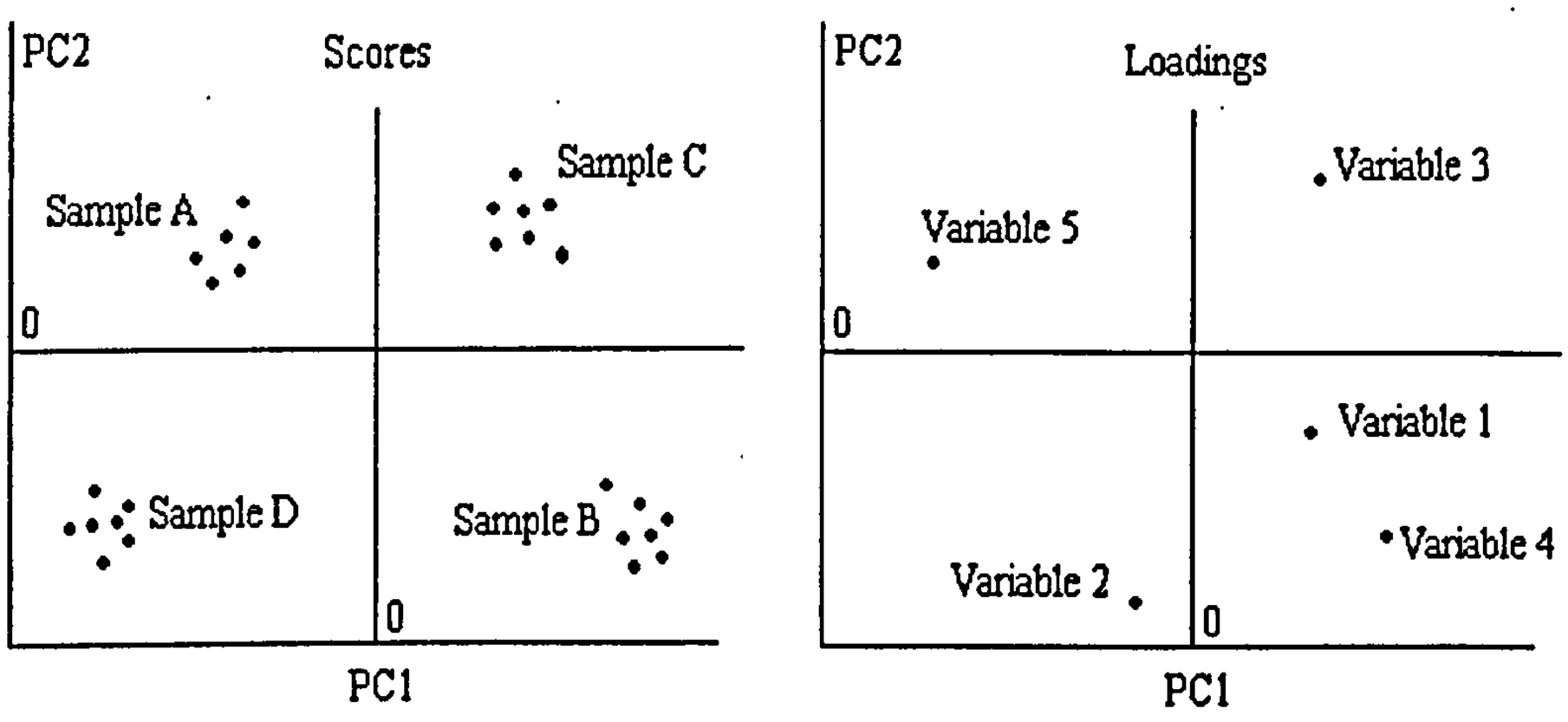


Figure 5.5: *Complementary Scatter Plots of Factor Scores and Loadings*

By comparing the plots of factor scores and loadings the actual variables which discriminate between the samples can be determined. Consider the example given in figure 5.5: it can be seen that sample A is identified by variable 5, sample B is identified by variables 1 and 4, sample C is identified by variable 3, whilst variable 2 identifies sample D.

5.3 Discriminant Analysis[116]

5.3.1 Introduction and Applications of Discriminant Analysis

As stated previously, discriminant analysis is a hard modelling technique which facilitates classification of a sample uniquely into one of a number of groups/classes by determination of the variables which discriminate between the groups. Thus, groups can be classified in accordance with the actual values of the discriminating variables.

In PCA the PC is the regression line which takes the direction of maximum variability through the data matrix. In contrast, the discriminant function (also known as the canonical variate) is the regression line through the data such that the difference between the groups is maximised.

The actual operating mechanism of discriminant analysis is canonical correlation analysis, whereby successive functions/roots which maximise discrimination between groups are determined. The first function provides the greatest discrimination overall, with each successive function providing decreasing levels of discrimination, as with successive principal components. Each function is orthogonal, *i.e.* independent.

5.3.2 Classification by Discriminant Analysis

Consider an unknown which must be assigned to a predetermined group 1, 2 or 3. It is necessary to elucidate the linear equation of variables which maximises the difference between the groups 1, 2 and 3. This linear equation is the discriminant function or canonical root and is of the form shown in equation 5.2.

$$\text{Canonical Root} = C + B_1V_1 + B_2V_2..... \qquad \textbf{Eq. 5.2}$$

where B_1, B_2 *et cetera* are the regression weights (also known as discriminant function coefficients) for variables V_1, V_2 *et cetera* and C is a constant. Each canonical root can be considered to be a product of the regression weights for each variable: the larger the weight, the more the variable contributes to the discrimination between groups. It does not, however, reveal which particular groups are discriminated. Eigenvalues are also obtained, as in PCA, the first canonical root having the largest eigenvalue.

For each canonical root the means for each root across the groups in the training set are calculated. The difference in the means for the groups for any root is related directly to the discriminatory powers of each canonical root. Consider the data in table 5.1:

Group	Means	
	Root 1	Root 2
A	-6.59	0.21
B	2.46	-0.77
C	4.06	0.32

Table 5.1: *Example Data Obtained for Discriminant Analysis*

For root 1 there is little discrimination between groups B and C, but group A is strongly discriminated from both B and C. Conversely, root 2 shows weaker discrimination of group B from A and C, though there is little discrimination between groups A and C.

Canonical root scores are obtained for each sample and, similar to factor scores in PCA, can be scatter plotted against the first and second roots, for example. Figure 5.6 represents a plot of the data in table 5.1.

The plot shows that root 1 strongly discriminates group A from groups B and C, whilst root 2 weakly discriminates between groups B and C.

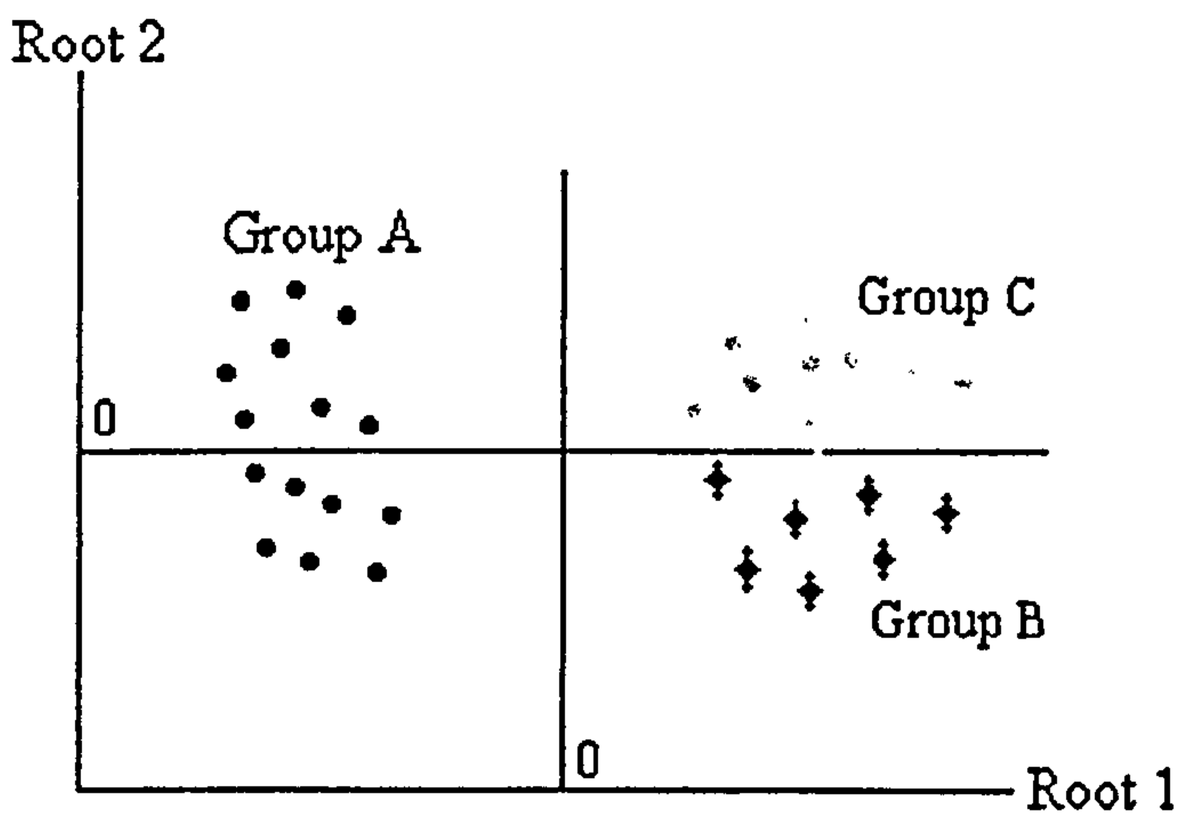


Figure 5.6: *Scatter Plot of Canonical Root Scores*

Factor structure coefficients are further statistical parameters obtained for each canonical root and are comparable to the factor loadings seen in PCA. Factor structure coefficients indicate the correlations between the variables in the model and the canonical roots.

A summary of these important statistical parameters and their functions is shown in table 5.2.

Parameter	Function
Regression Weight Coefficient	Shows contribution of each variable to canonical root
Canonical Root Scores	Show discrimination between groups
Means of Canonical Root Scores	
Factor Structure Coefficient	Allows interpretation of between group discriminatory roots in terms of variables

Table 5.2: *Summary of Parameters and Their Functions*

Once these important statistical parameters have been extracted from the training set, the model can be used for the classification of unknown samples. The process is illustrated diagramatically in figure 5.7.

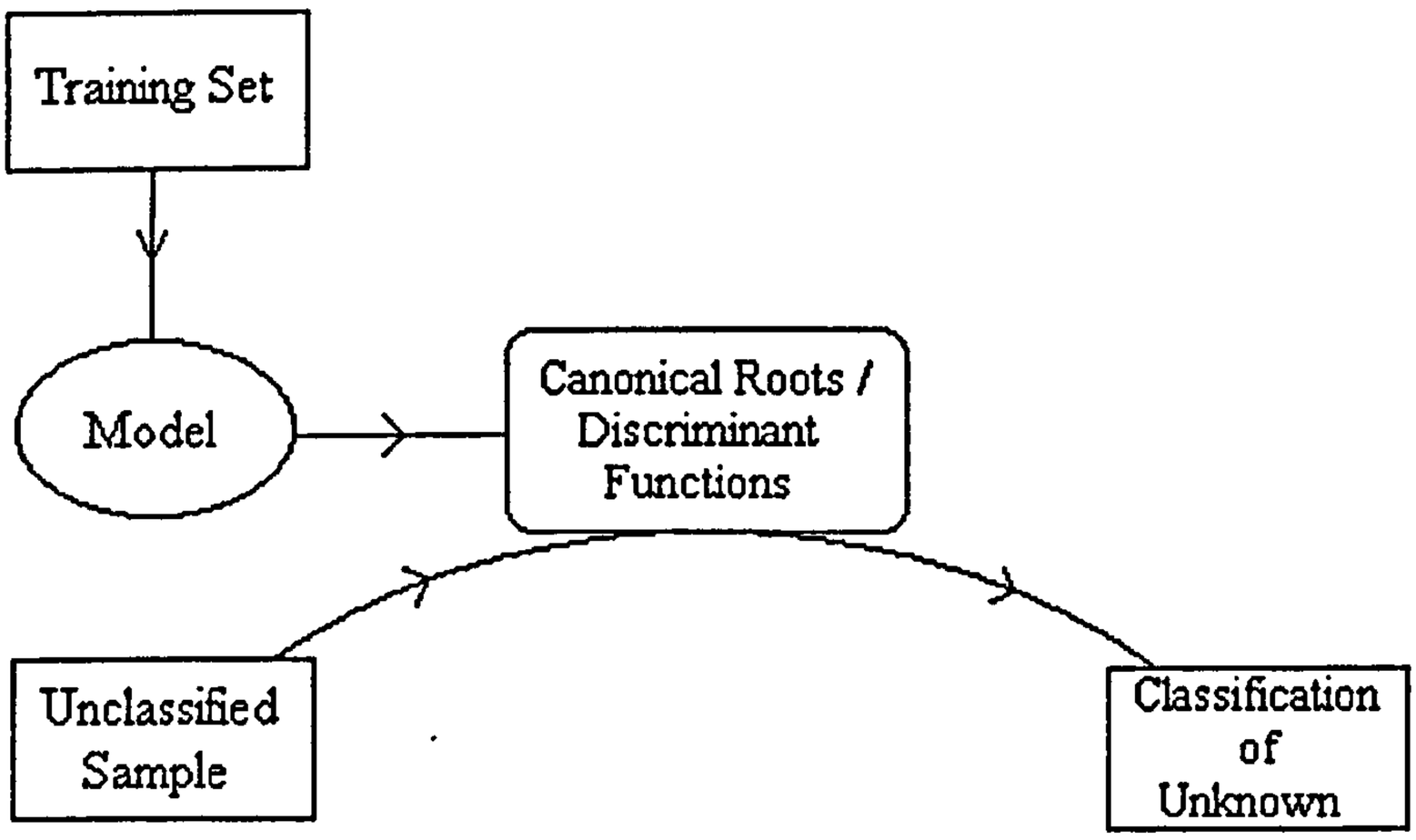


Figure 5.7: *Schematic Diagram Illustrating Classification Process*

Classification of unknown samples is achieved by calculating their classification scores, from the regression weights and the observed values of the discriminating variables, using equation 5.3:

$$S_{group\ 1} = C_{group\ 1} + R_{V1} * V1 + R_{V2} * V2.... \qquad \textbf{Eq. 5.3}$$

where $S_{group\ 1}$ is the classification score of a particular sample for a particular group (e.g. egg yolk), $C_{group\ 1}$ is the group constant, R_{V1} is the regression weight for variable 1 in group 1, V1 is the value of variable 1 *et cetera.* Discriminant function software generally provides such information as "classification functions" (simple linear equations of the type shown in equation 5.3), which allow facile determination of the group membership of unknown samples. An unknown sample is classified as belonging to that group for which its classification score is highest.

5.4 Use of Pattern Recognition Techniques for Classification of Results

As stated previously, both PCA and discriminant analysis were used to assist in the classification of suspected mixed media samples.

5.4.1 Classification of Mixed Proteinaceous Media by Discriminant Analysis

A total of seventy two samples of a variety of known standard proteinaceous media were analysed, providing a large training set for discriminant analysis. The various types of standard media comprised the groups to be discriminated, resulting in a model for the classification of the suspected mixed media samples.

Table A1 (appendix 5) shows the data used in the training set and the group classifications. The data cannot be processed if any variable equals zero, so extremely small values (in the order of 0.001) which would not interfere with any trends in the variables were substituted where appropriate – this was also done for the experimental data obtained for those samples which were thought to contain a mixture of proteinaceous media.

The data was processed using *Statistica* (Stat-soft), a computer package for pattern recognition, and the parameters for discriminant analysis were obtained. Regression weights and constants for the training set data are shown in table A2 (appendix 5) whilst table A3 (appendix 5) contains details of the means of the canonical roots.

Once the discriminant analysis parameters had been obtained, it was possible to calculate the classification scores for each group for each of the unknown samples. The higher the group classification score of a particular sample, the greater the probability that the sample belongs to that group: samples which were suspected of containing a mixture of proteinaceous media typically displayed high scores for a number of groups, indicating the probable presence of each media type in the mixture. Test samples of known identity, comprising both pure and mixed media, were used in order to prove the suitability of discriminant analysis for the classification of samples of this type. The experimental data obtained for the test samples is shown in table A4 (appendix 5).

Scatter plots of the canonical root scores (from equation 5.2) are shown in figures 5.8 and 5.9. The scatter plots of root 1 against root 2 and root 1 against root 3 clearly show strong discrimination between the groups

egg yolk, milk, egg albumin and the animal/fish glue media. Both plots show weaker discrimination between the egg albumin and casein groups, though the plot of root 1 against root 2 does have a slightly clearer separation between the two groups.

The classification scores for the test samples, shown in table A5 (appendix 5), confirm the suitability of the discriminant analysis method for the classification of mixed media samples. As expected, the highest classification scores (shown in bold type) were seen for the actual proteinaceous media used in the test samples, *i.e.* test sample 4 had the highest scores for egg albumin and casein/milk, whilst the highest scores for test sample 1 were those of the animal/fish glue media.

It was observed in test samples 5 and 8 that the score for egg albumin was almost exactly the same as that for casein: this result is not unexpected when studied in conjunction with the previous scatter plots, both of which showed only slight discrimination between the egg albumin and casein groups. However, sample 5 can be confirmed as a pure casein medium since the score for the milk group (*i.e.* casein from a different supplier) is far greater than the score for either the egg albumin or casein groups.

Tables A6 (appendix 5) and A7 (appendix 5) contain the experimental data and classification scores obtained for the suspected mixed media samples. The statistical summary for the discriminant analysis of experimental results obtained from the analysis of proteinaceous media samples is shown in table A8 (appendix 5).

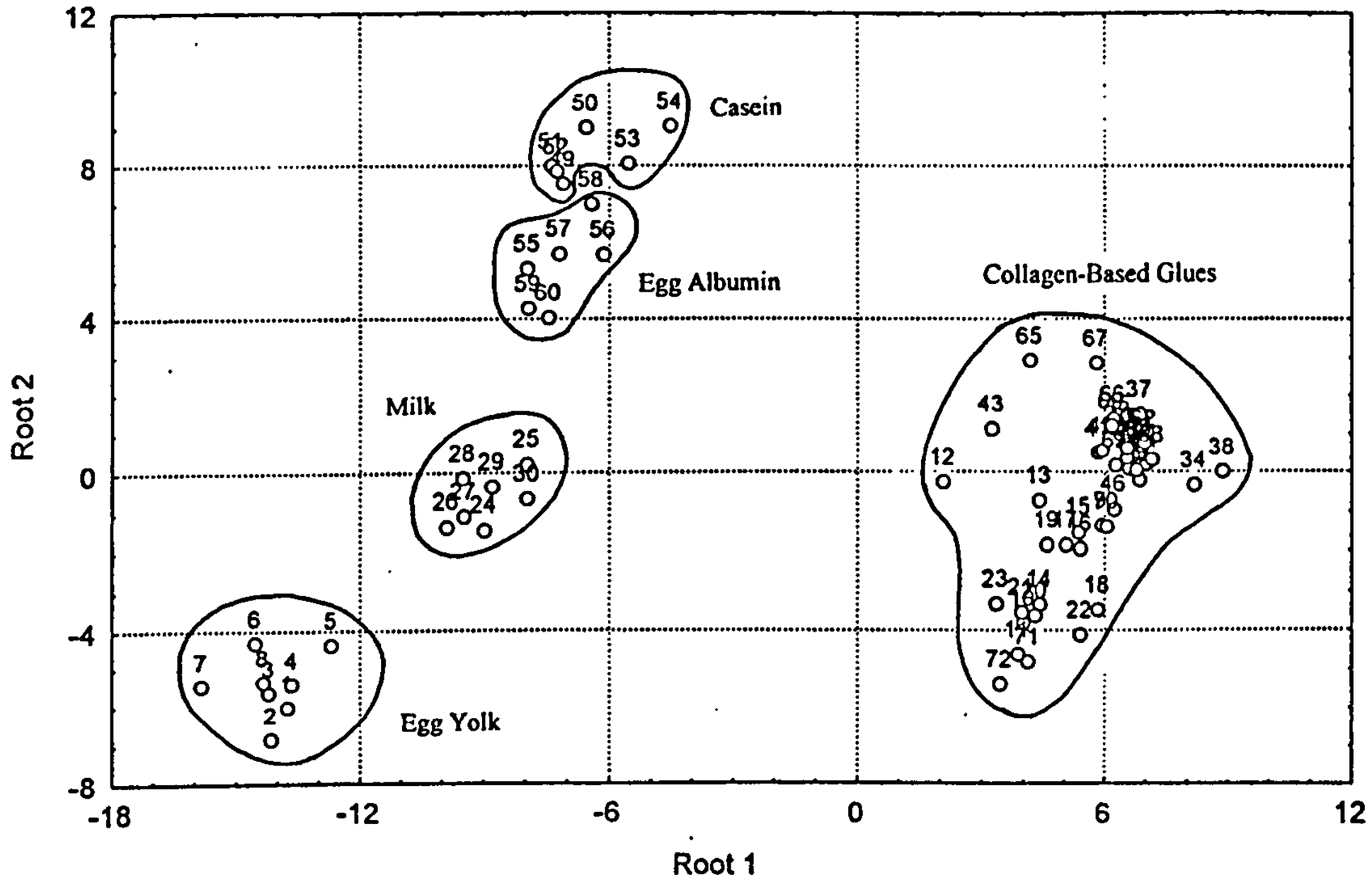


Figure 5.8: *Scatter Plot of Root 1 Against Root 2*

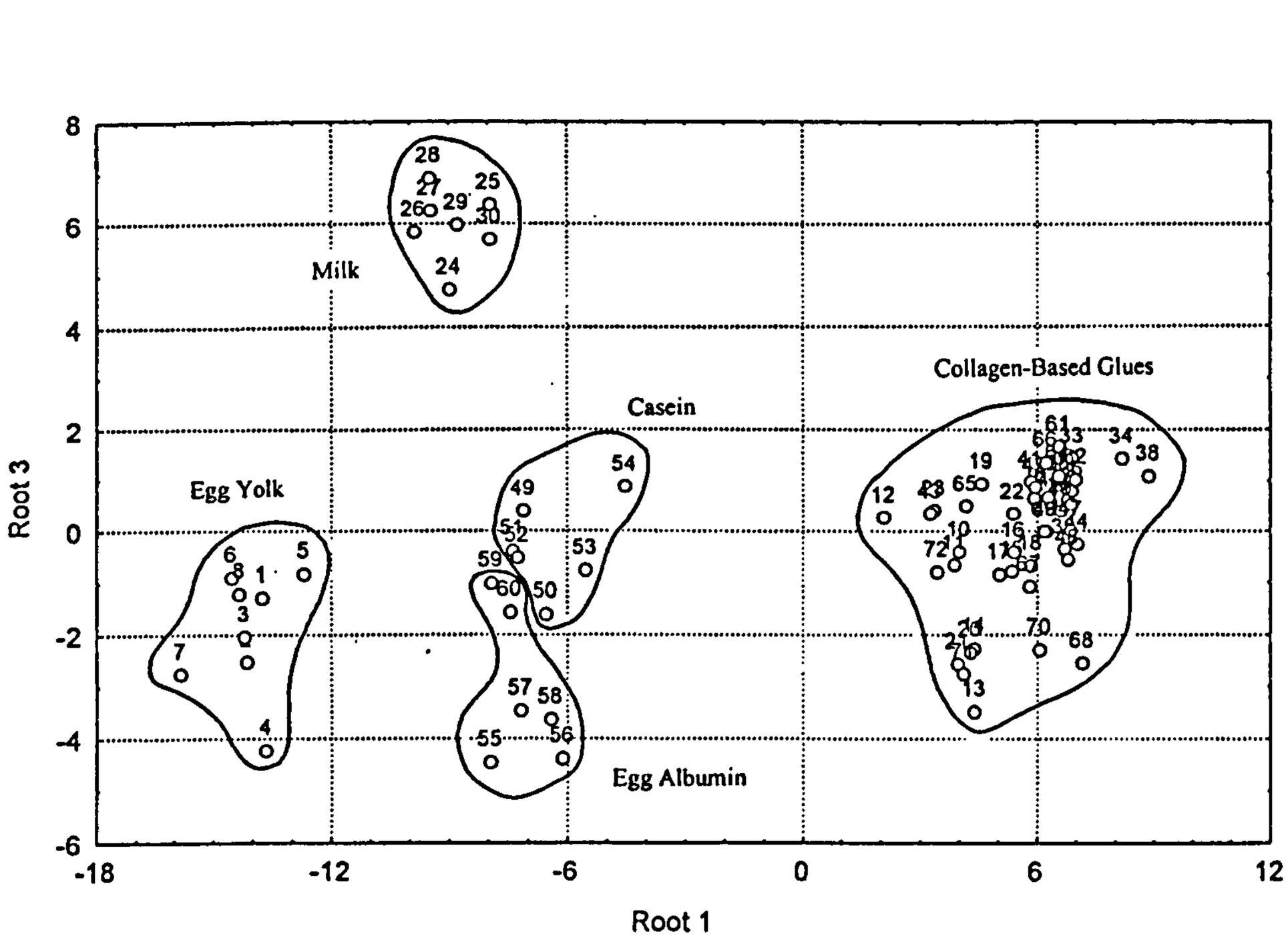


Figure 5.9: *Scatter Plot of Root 1 Against Root 3*

The specified groups comprising the model for classification purposes are described in table 5.3.

Group	Media Type
Yolk	Hens' egg yolk
Glue	Animal glue
Fish	Isinglass
Milk	Casein (supplier1)
Strong	High strength skin glue
Skin	Skin glue
Bone	Bone pearl glue
Casein	Casein (supplier 2)
Albumin	Hens' egg albumin
Gelatine	Gelatine sheet
Parchment	Parchment for glue making (from deer)
Sturgeon	Sturgeon glue

Table 5.3: *Key to Group Descriptors*

5.4.2 Conclusions from Discriminant Analysis for Proteinaceous Media Samples from Works of Art

The training set data used and discriminant analysis results obtained for proteinaceous media can be found in appendix 5, table A1 to A7.

The sample of priming from Turner's *George IV's Departure from the 'Royal George'* (N02880) was concluded to be a mixture of casein and egg albumin, since the highest classification scores were observed for the casein/milk and egg albumin groups.

The same conclusion regarding the identity of the medium was drawn from the results obtained for the priming sample removed from a second work by Turner, *Judith with the Head of Holofernes* (N05500). The highest classification scores were seen for the egg albumin and casein/milk groups.

The composition of the adhesive from Rossetti's *The Tune of the*

Seven Towers (N03059) was also elucidated. The highest classification scores were seen for the egg yolk and fish/sturgeon/glue groups, hence it was concluded that Rossetti's adhesive was comprised of egg yolk and a collagen glue, most likely of fish origin.

The priming samples removed from Ketel's series of family portraits, *Alice Smythe*, *Robert Smythe* and *Joan Smythe*, were thought contain an animal glue and egg yolk medium. The presence of both was confirmed but, for samples taken from *Robert* and *Joan Smythe*, the egg yolk group had the highest classification score, next to fish/sturgeon glue, whilst the reverse was true for *Alice Smythe*. It was concluded, therefore, that the priming medium was comprised of egg yolk and glue, though the proportions probably varied over the series thus giving rise to the differences in classification scores seen for the three works.

The priming of Reynold's work *Sir James Hodges* (N03545) most probably contains a mixture of whole egg, casein and a small amount of fish glue. The highest classification scores were seen for the groups egg yolk, albumin and casein, whilst the fish/glue groups had a slightly lower score which was still noticeably higher than those of the remaining groups. Since a significant level of hydroxyproline was found in the sample, a collagen glue must be present.

Finally the coating removed from *The Rescue of Andromeda* (N01749), a bronze sculpture by Fehr, was concluded to be a mixture of a collagen glue (probably of fish origin) and egg yolk since these groups had the highest classification scores.

5.4.3 Investigation of Gum Media Using PCA and Discriminant Analysis

Since the chromatographic method used for the analysis of the gum media samples was not quantitative, the sample weights being unknown, it was necessary to attempt some form of standardisation of the chromatographic data in order to perform PCA and discriminant analysis. Galactose is present in all the gum media studied, hence it was decided to standardise the peak heights with respect to galactose and, in the standard samples, to adjust the heights according to the percentage galactose quoted in the literature.

For each sample, the peak height of galactose was measured manually, using the millimetre scale of an ordinary ruler, then the peak height of each of the other monosaccharides was divided by the galactose peak height to give an adjusted value *x*. The multiplication factor for standardisation was calculated as follows: it has been reported that gum arabic contains 49.47 % galactose,[78] whilst gum tragacanth contains only 11.46 % galactose.[78] Dividing one by the other gave a multiplication factor of 0.232 – this factor was then multiplied by *x* to give a standardised peak height with respect to galactose. Each of the factors was calculated relative to gum arabic (49.47 % galactose was designated as equivalent to a peak height of 1). Table 5.4 shows the multiplication factors used for the peak height standardisations of each of the standard gum media types.

Table A9 (appendix 5) contains details of the standardised chromatographic data comprising the training set and group classifications. As mentioned previously in section 5.4.1, the data cannot be processed if any

variable equals zero, thus negligible values (in the order of 0.0001) were substituted where appropriate.

Gum	% Galactose[78,92]	Multiplication Factor
Arabic	49.47	1.000
Tragacanth	11.46	0.232
Cherry	21.00	0.424
Karaya	32.88	0.665
Ghatti	41.92	0.847
Guar	34.23	0.692
Locust Bean	20.76	0.420

Table 5.4: *Galactose Composition and Multiplication Factors for Peak Height Standardisation*

Each numbered variable in table A9 corresponds to a peak whose retention time is shown in parentheses. The identity of every peak is not known, primarily due to the complexity of the chromatograms, though most of the major peaks have been characterised.

PC Value	Eigenvalue	% Total Variance	Cumulative Eigenvalue	Cumulative % Variance
1	11.975	41.292	11.975	41.292
2	6.623	22.839	18.598	64.131
3	4.029	13.893	22.627	78.024
4	2.899	9.998	25.527	88.022
5	1.087	3.749	26.614	91.772
6	1.048	3.614	27.662	95.386

Table 5.5: *Eigenvalues and Cumulative Percentage Variance for Principal Components*

The data was processed using the *Statistica* package, as before. The scree plot (figure 5.10) identified six principal components and table 5.5 shows the eigenvalues and cumulative percentage variance associated with the principal components.

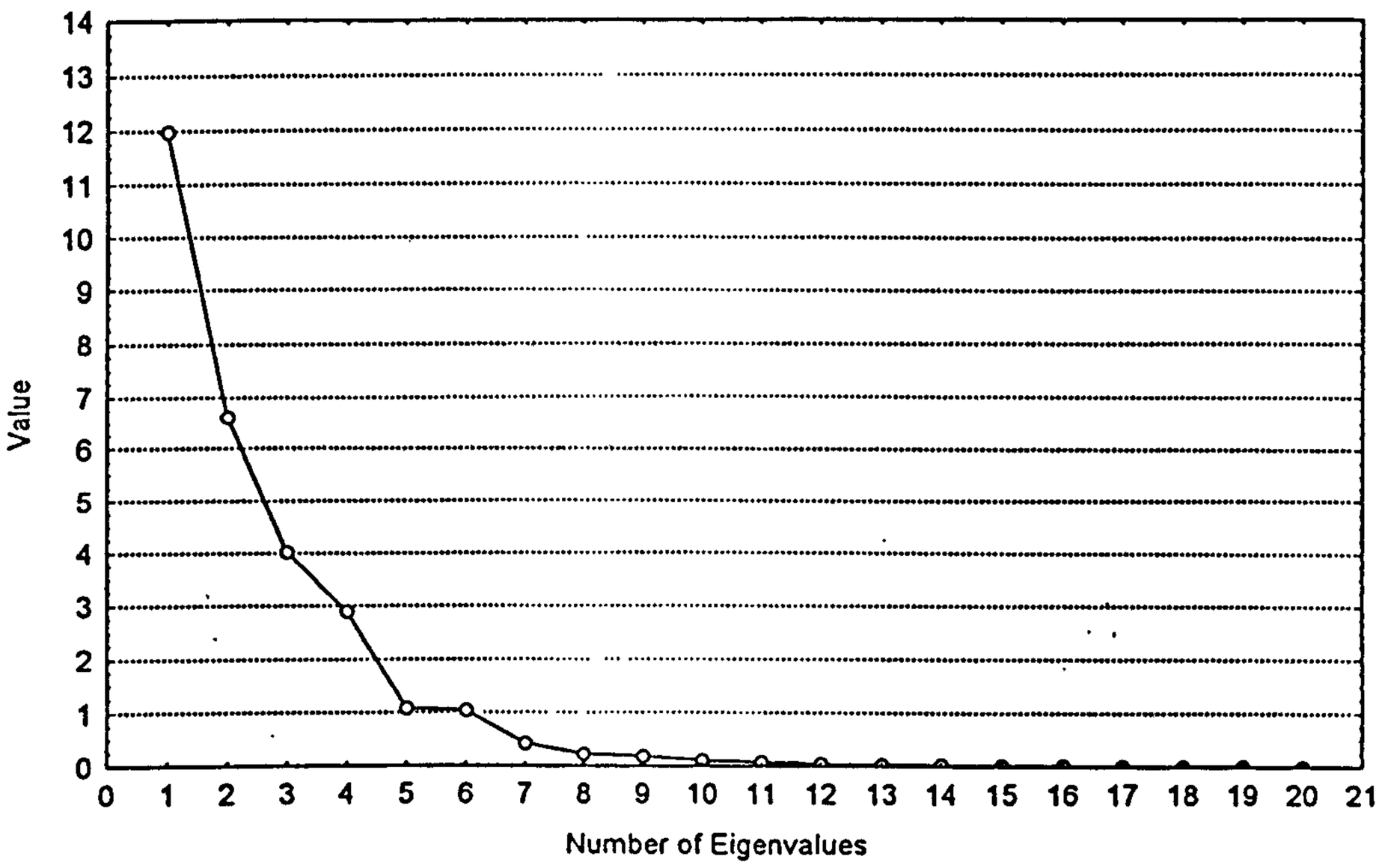


Figure 5.10: *Scree Plot of Eigenvalues from PCA*

A selection of scatter plots of factor scores is shown in figure 5.11. All the plots show a reasonable level of discrimination between the media types, though some groups are slightly better discriminated than others. Consider the plot of factor 2 (PC 2) against factor 1 (PC 1): these principal components show greatest discrimination between gums arabic and karaya, which are clearly grouped, whilst guar and locust bean are slightly less

distinct and ghatti, cherry and tragacanth are considerably more difficult to separate. Therefore, PC 1 and PC 2 are most significant for the arabic gum and the karaya gum groups respectively. Now look at the plot of factor 2 (PC 2) against factor 3 (PC 3): again gum karaya is well separated, along with locust bean and guar, whilst ghatti has become more strongly discriminated than in the previous plot. However, gum arabic has become less distinct, falling close to the tragacanth and cherry gum groups. These observations confirm that PC 2 is

highly significant for the karaya gum group and indicate that PC 3 has more significance for the ghatti gum group. Finally, the plot of factor 2 (PC 2) against factor 4 (PC 4) shows probably the clearest separation between all the groups, indicating that these particular principal components are reasonably significant to each of the groups.

Discriminant analysis was also performed on the training set data shown in table A9, though unfortunately proved to be less successful than for the classification of the proteinaceous media samples. Table A10 (appendix 5) shows the regression weights and constants obtained for the discriminating variables in each of the groups.

Plotting the means of the canonical roots 1 and 2 for each group resulted in the scatter plot shown in figure 5.12. There is very strong discrimination between all seven of the groups: root 1 provides maximum discriminating power for the groups tragacanth, arabic and karaya, whilst root 2 discriminates more strongly between the groups guar, locust bean and ghatti. The cherry gum group lies somewhere in the middle of the others.

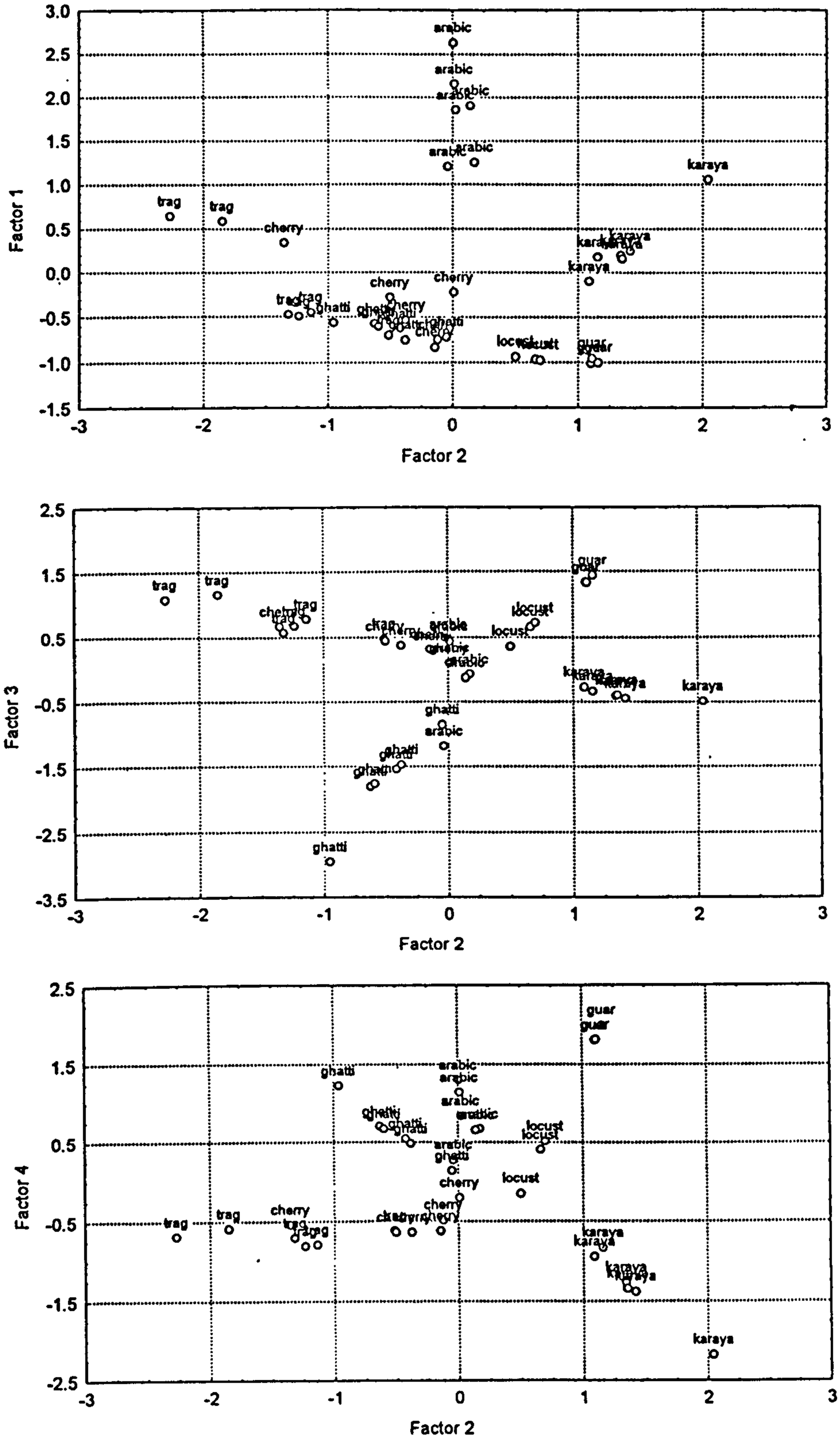


Figure 5.11: *Scatter Plots of Factor Scores for Classification of Gum Media*

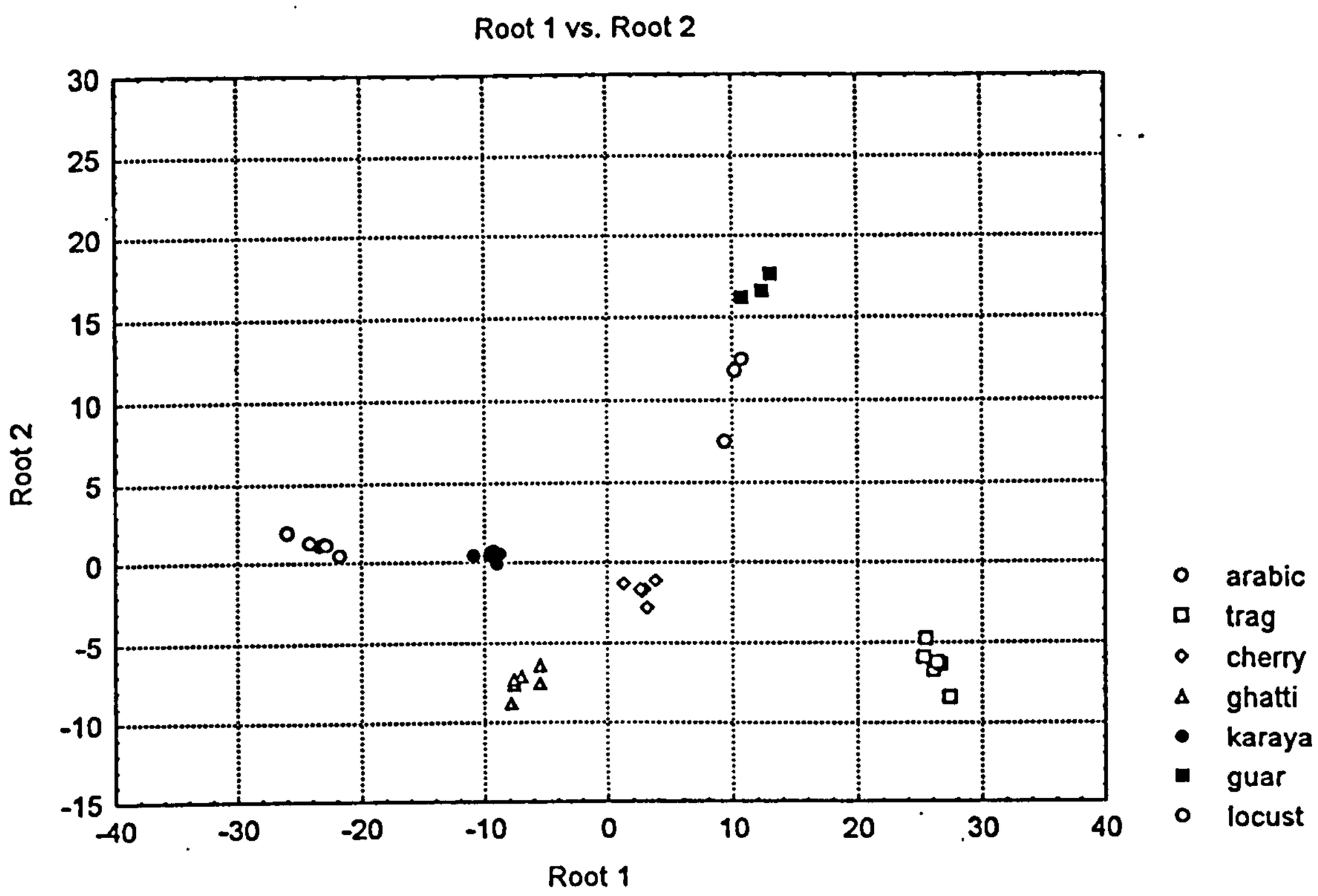


Figure 5.12: *Scatter Plot of Canonical Roots*

However, standardisation of chromatographic results obtained for the unknown samples was obviously impossible, since the relevant multiplication factor could only be found when the identity of the sample was known. Thus, when discriminant analysis was tested using mixed samples of known composition, it was found that the resulting answers were incorrect. When the classification scores were calculated for the test samples the results were not as expected, *i.e.* the highest scores were not necessarily obtained for those

components known to be present in the mixtures. Therefore, at this stage, it was not feasible to apply the method to those unknown samples suspected of containing a mixture of gum media. The most likely explanation for this is, as mentioned previously, the fact that the chromatographic method is only qualitative and that the attempts to standardise the data to reflect a quantitative analysis are not viable. To enable quantitative analysis, it will be necessary to analyse standard samples of a known mass, including a suitable internal standard, in order that the response can be related to the amount of gum medium present in a sample. Once a truly quantitative method has been established, it is likely that discriminant analysis will prove useful in the classification of suspected gum media samples removed from works of art.

CHAPTER 6

Discussion and Conclusions

The experimental procedures described in Chapter 3 facilitated the analysis and subsequent identification of samples of proteinaceous and gum media, used by artists as pigment binders, adhesives, grounds and sizing materials. The analysis of standard protein and natural gum sources, in order to confirm differences in amino acid ratios and monosaccharide compositions respectively, revealed chemical "fingerprints" for each substance, thus enabling their differentiation.

The identification of the media used by the artists in the particular works under investigation facilitated the study of the techniques and materials favoured by these artists. Conclusions regarding the relationship between artist and materials and materials and condition of the works could then be drawn.

6.1 Methodology for Analysis of Proteinaceous Media

6.1.1 9-Fluorenylmethyl Chloroformate as a Derivatising Agent

A number of contributory factors influenced the selection of 9-fluorenylmethyl chloroformate (FMOC) as the derivatising agent for amino acid analysis: FMOC provides a reproducible derivatisation technique yielding stable amino acid derivatives, plus the choice of a variety of highly sensitive methods suitable for the detection of the minute levels of proteinaceous material present in samples removed from works of art.

Firstly, the reaction of FMOC with primary and secondary amino acids is rapid, complete derivatisation being achieved in only 30 to 40 seconds. Secondly, the reaction favours mild aqueous conditions and the derivatised product yield is typically reported to be high, around 89 %.[117, 118]

Furthermore, the FMOC moiety is both a good UV chromophore and highly fluorescent, allowing a choice of emission or absorption detection techiques: the FMOC-amino acid derivatives can be detected at limiting levels in the low femtomole range by excitation at 260 nm.[119]

The mechanism of the nucleophilic substitution reaction between FMOC and the amino acid residues is detailed in figure 6.1. FMOC will also react with the water present in the reaction mixture, to form the corresponding alcohol as a hydrolysis product.[119] The extent of any undesirable hydrolysis products is minimised by the prompt extraction of the reaction mixture with hexane or pentane: it is worthy of note that the depletion of the derivatising agent as a result of any hydrolysis product formation is insignificant. A more polar solvent (*e.g.* chloroform) should not be used for the extraction, since this can result in up to a 75 % loss of the more hydrophobic amino acid derivatives, *e.g.* lysine, into the discarded organic layer, leading to irreproducible results.[120]

6.1.2 Stability of FMOC-Amino Acid Derivatives

Stability studies have shown that FMOC-amino acid derivatives are stable at room temperature and in daylight for at least two weeks.[121] Standard samples of 16 derivatised amino acids (hydroxyproline, glutamic acid, alanine, serine, methionine, aspartic acid, valine, glycine, proline,

phenylalanine, leucine, tryptophan, isoleucine, arginine, lysine and threonine) were stored in darkness and daylight, at 4 °C and at room temperature respectively, for a period of 2 weeks, during which time the samples were periodically analysed to monitor any degradation: there were no signs of any significant decomposition of any of the derivatives in the standard sample mixture.

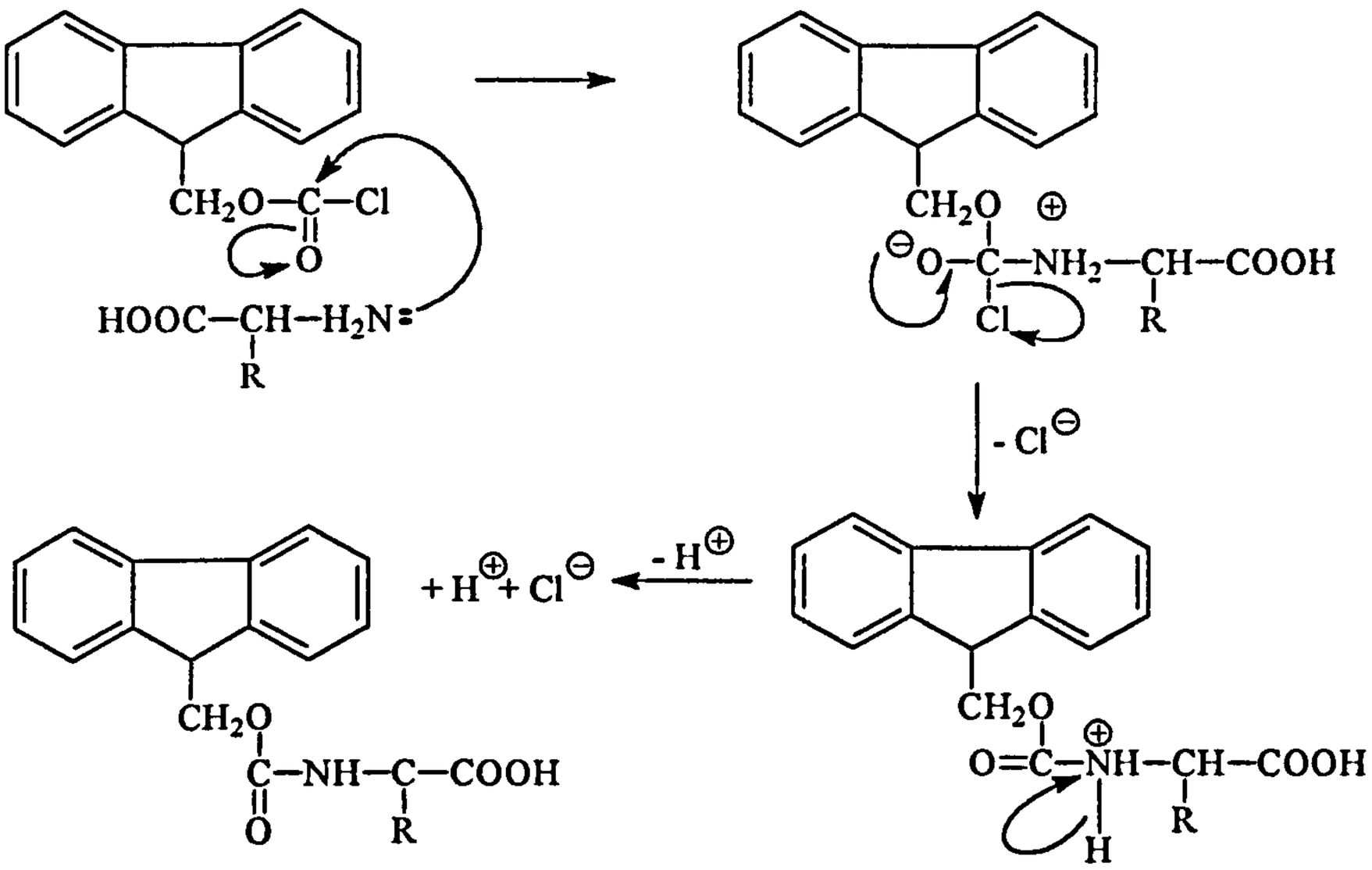


Figure 6.1: *Mechanism of FMOC Reaction with Amino Acids*

6.1.3 Separation and the Selection of the Mobile Phase

The samples can be represented by the equilibrium:

$$R\text{-}COOH + H_2O \rightleftharpoons R\text{-}COO^{\ominus} + H_3O^{\oplus}$$

The samples can be considered as weak acids and as such would preferentially partially dissociate to yield $RCOO^-$ ions: these would be

virtually unretained on the column due to their high polarity. The use of a buffered mobile phase controls this dissociation, keeping the equilibrium to the left, therefore facilitating adequate retention of the less polar R-COOH molecules. Also, the presence of triethylamine in the buffer ensures speedy elution of the arginine derivative and reduces any peak tailing.[119]

Preliminary investigations into the suitability of an acetate buffer/acetonitrile mobile phase revealed the presence of a large interfering absorbance which masked some of the amino acid derivative peaks. Further investigations into the pH of the buffer and samples for instance, indicated that the problem lay with the mobile phase itself, rather than the samples. The interference was almost completely eradicated by using gradient grade HPLC solvents.

6.1.4 Pigment Interferences

Derivatisation of the egg tempera paint samples containing azurite (basic copper carbonate) and verdigris (basic copper acetate) was initially unsuccessful. This was undoubtedly due to the presence of copper in the pigments, as the copper (II) ions form a chelation complex with the amino acids (figure 6.2).[122]

The addition of the FMOC moiety is prevented by this chelation because the amino group is unable to take part in any reaction. By reacting the buffered sample solution with aqueous ethylenediaminetetraacetic acid (EDTA, disodium salt, dihydrate) prior to derivatisation the copper-amino acid chelation is prevented, since EDTA preferentially complexes with the copper ions.

Section 3.1.2.1 describes how an aliquot of EDTA solution was added to samples which contained copper-based pigments: derivatisation and subsequent analysis of the samples was then successfully achieved.

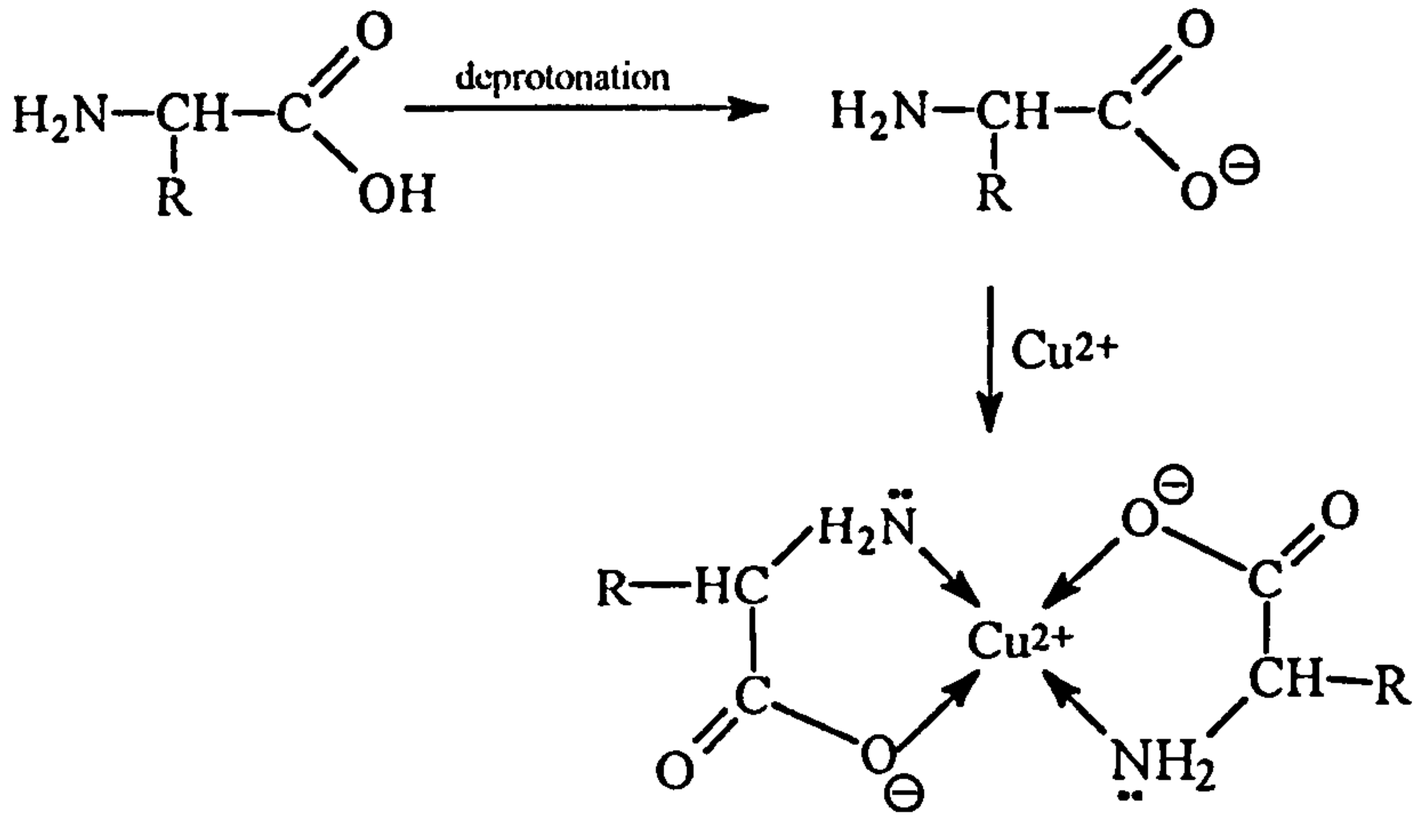


Figure 6.2: *Copper - Amino Acid Chelation Complex Formation*

6.1.5 Investigations into Egg Media

As mentioned earlier in chapter 4, section 4.1.1, Grzywacz[112] investigated the amino acid compositions of eggs from a variety of species and locations, in order that any possible differences in their composition could be used for identification purposes. Various eggs from North America, Europe, the Far East and New Zealand were analysed in the course of her study. The results showed only slight variations in the composition of eggs from different locations, which were within the limits of variation due to the actual analytical method itself: it was concluded that egg from different geographical locations could not feasibly be distinguished *via* this method. Very subtle differences were seen in the compositions of eggs from hens,

ducks and geese (where the whole egg was analysed) but, unless the exact species of egg was required, it is acceptable to assume that hens' eggs would be a suitable standard for comparison with any suspected egg medium.

6.2 Methodology for Analysis of Natural Gum Media

6.2.1 Silylation of Carbohydrate Compounds for GC Analysis

6.2.1.1 Mechanistic Aspects of Silylation

Silylation is a versatile technique used to increase the volatility of carbohydrate compounds for GC analysis: this is achieved *via* blockage of the active proton sites, which results in a reduction of dipole-dipole interactions. Mass spectrometric properties of a compound may also be improved by silylation, leading to clearer fragmentation patterns and the introduction of characteristic ions useful in trace analyses.

Silylation of many of the protic functional groups found in natural and synthetic organic compounds is easy, hence the popularity of this particular derivatisation technique. The silylation reaction itself is relatively simple, involving the displacement of the active proton from the functional group (*e.g.* OH, SH, NH, COOH *et cetera*).[123] The general reaction for the formation of a trialkylsilyl derivative is shown below:

$$R_3Si{-}X \;+\; R'{-}H \longrightarrow R_3Si{-}R' \;+\; HX$$

The most common silylating agents of the trialkylsilyl type possess the methyl group, yielding trimethylsilyl (TMS) derivatives. Mechanistically,

the silylation reaction can be considered as nucleophilic substitution, resulting in the formation of a bimolecular transition state (figure 6.3).

The nature of the leaving groups X and Y must be considered when selecting a suitable silylating agent. The leaving group Y should be easily lost from the transition state during the reaction and the leaving group X must have low basicity coupled with the ability, whilst in the transition state, to stabilise a negative charge: there should also be little or no π(p-d) back-bonding between the silicon atom of the silylating agent and the leaving group, X.[124,125]

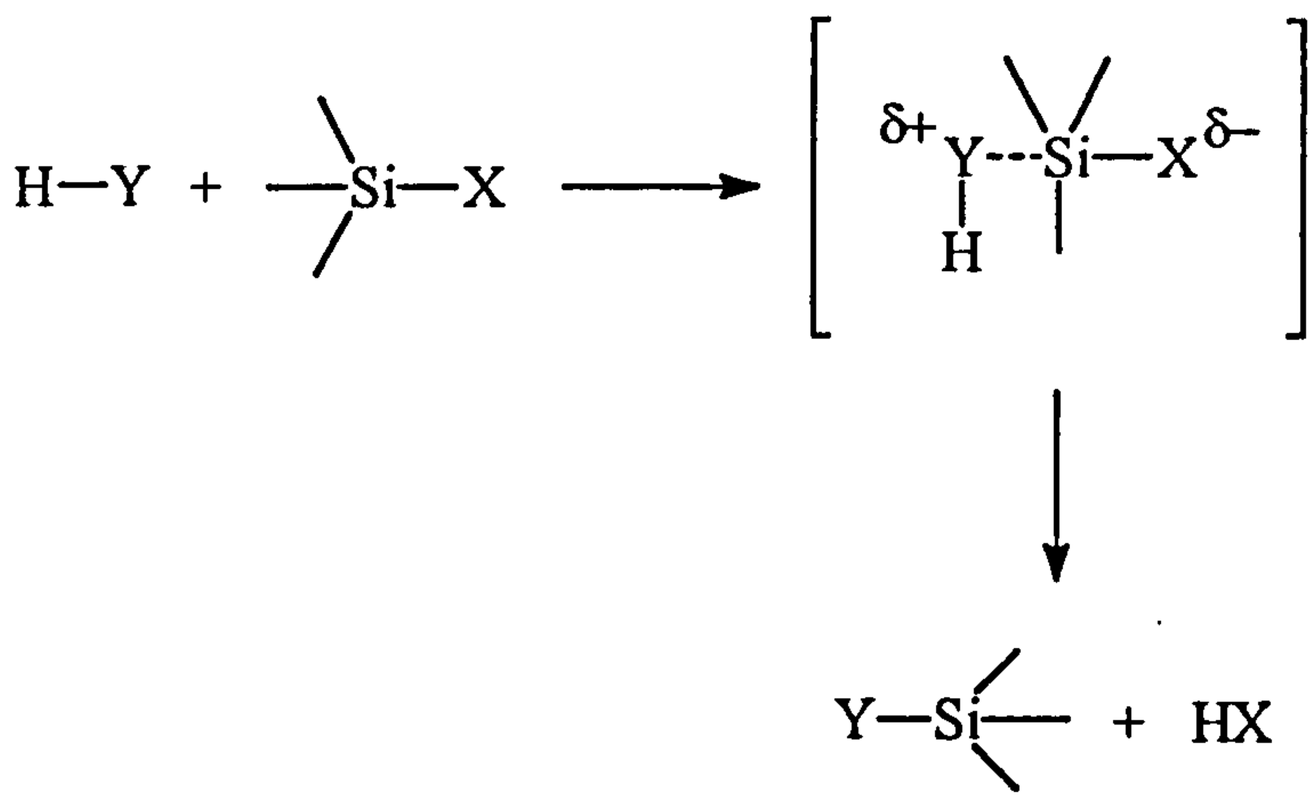


Figure 6.3: *Formation of Bimolecular Transition State*[126]

The formation of the transition state is reversible, thus complete silylation will only occur if the basicity of the leaving group Y is less than that of X. Investigations have revealed a high degree of correlation between the basicity of leaving group Y and the donor strength of *N*-silyl derivatives.[124,127]

For a given silylating agent, the ease of silylation of protic functional groups is in the order alcohol > phenol > carboxylic acid > amine > amide. Within the groups of the series reactivity is determined by steric hindrance, thus primary alcohols are more reactive than either secondary or tertiary alcohols *et cetera*.[128]

6.2.1.2 Practical Aspects of Silylation

The primary problem associated with silylation is that all silylating reagents and the resulting silyl derivatives are particularly susceptible to hydrolytic attack by any moisture present in the reaction mixture, ultimately leading to incomplete derivatisation.[129] Trimethylsilyl (TMS) derivatives are especially sensitive, more so than silylated derivatives which possess a much more sterically hindered silicon atom: water contamination as low as 0.2 % during trimethylsilylation resulted in a considerable loss of all sugar residues.[130] Despite this, the trimethylsilylation of aqueous solutions of hydroxy compounds has been achieved, using a great excess of derivatising agent.[131] Chemical and hydrolytic stability of silyl derivatives is increased by steric hindrance, resulting in increased sample recovery post work-up and even facilitating the use of water-washing stages in the preparation of samples.[132,133]

In most cases the silylating agent alone is an adequate solvent, but sometimes an additional solvent is required in the reaction mixture and for sample dilution prior to analysis. The selection of a solvent is crucial since any active hydrogens (*i.e.* those of the solvent in addition to those present in the compound to undergo derivatisation) will be silylated. For this reason,

and also because of its catalytic properties and ability to act as an HCl acceptor in reactions involving organochlorosilanes, pyridine is an ideal solvent for silylation reactions.[134,135] Where an additional solvent is added to the derivatisation mixture, the solubilisation of the analytes prior to silylation may not be entirely necessary, since this may occur as the silylation reaction itself proceeds. Heating is often used to facilitate efficient silylation: the thermal stability of analytes and derivatising reagents should be considered under these conditions. If a sample requires further dilution after derivatisation, the accepted practice is to use an inert solvent such as hexane.

In the ideal situation silyl derivatives would be produced quantitatively under mild conditions, giving rise to a single peak for each analyte *via* GC analysis. However, as discussed above, the silylation reaction is subject to variation under certain conditions – the problems caused by moisture can be minimised by the use of a large excess of derivatising agent or solvent, whilst improved manufacturing practices mean that impurities in the reagents which could mask analyte components are negligible.[136]

One problem inherent in GC analyses of silylated compounds is the accumulation of SiO_2 in the flame ionisation detector, as a by-product of the combustion of any residual silylating agent: removal of excess reagent prior to analysis minimises this effect.[137] However where extraction procedures are unfeasible, such as in trace analyses, and a reasonable amount of silylating agent is repeatedly injected, a build-up of SiO_2 is inevitable. Since simply increasing the detector temperature does not ensure removal of the deposits, a regular thorough cleaning of the detector is the best way to maintain optimum detector performance.

6.2.1.3 Hexamethyldisilazane as a Silylating Agent

Hexamethyldisilazane (HMDS) was amongst the earliest available silylating agents,[138] though it is not as strong a silyl donor as some other reagents. However, HMDS can react selectively and its silylating power can be enhanced by the catalytic presence of trimethylchlorosilane (TMCS) in the reaction mixture.[139]

Mixtures of HMDS/TMCS in pyridine are widely used for the silylation of carbohydrate materials,[140-143] though any precipitated ammonium chloride could result in column contamination.[144] Extraction of the reaction mixture with hexane removes the contaminant but, if very small volumes are used, direct injection of the sample can be performed.[145] All free hydroxyl groups are silylated and yields of the TMS derivatives are virtually quantitative.[69]

Generally there is no need for an additional solvent, since HMDS is liquid at room temperature and has adequate solvating properties for most classes of analyte.

A method of sample preparation using HMDS, with trifluoracetic acid in pyridine, has been developed for the silylation of syrups and concentrated aqueous solutions of sugars and starch hydrolysates[146] – it was this particular method which was adapted for the analysis of gum media samples removed from works of art. The procedure proved highly satisfactory, facilitating rapid derivatisation of the gum hydrolysates. The reagents may not be prepared in advance and caution should be exercised, since the reaction is highly exothermic and ammonia gas is evolved.

Good separation of the TMS derivatives can be achieved using isothermal conditions for samples with a small range of molecular weights, though a linear temperature gradient is necessary for samples which contain analytes with a wider spread of molecular weights. Anomers and structural isomers may also be separated.[69]

6.2.1.4 Trimethylsilylimidazole as a Silylating Agent

Trimethylsilylimidazole (TSIM) is generally regarded as the strongest silylating agent for hydroxy compounds, although it is unreactive towards aliphatic amines.[139,147] The low volatility of TSIM compared to many other common silylating reagents means the direct injection of TSIM onto capillary columns is inadvisable. The reaction between TSIM and the monosaccharide arabinose is shown in figure 6.4.

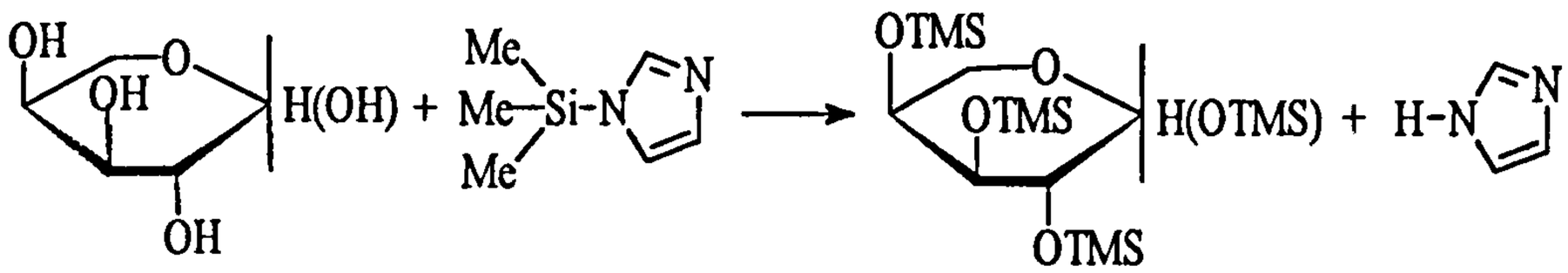


Figure 6.4: *Silylation Reaction Using TSIM*

Imidazole and hexamethyldisiloxane are produced by the rapid decomposition of TSIM in moist air, which requires careful handling during sample preparation.

Analytes which possess a keto group, such as carbohydrates, form enol-TMS ethers which are undesirable since their formation is not quantitative.[148] The formation of enol-TMS ethers can be prevented by using TSIM: since TSIM is a strong silyl donor, quantitative derivatisation can be achieved without using acid catalysts which promote enol-TMS formation. The imidazole by-product does not encourage enol-TMS ether formation, due to its weakly amphoteric nature.[149]

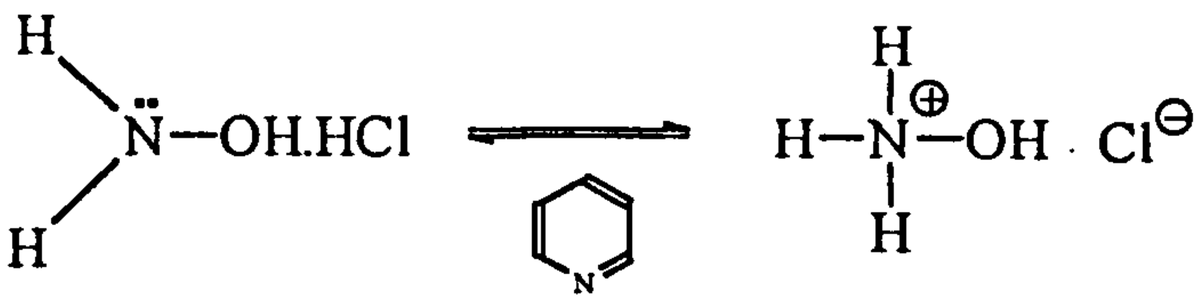
6.2.2 Use of Oxime Derivatives

One problem with the silylation of carbohydrates is the existence of multiple reaction products, resulting in chromatograms which are complex and difficult to interpret. The multiple products yielded by monosaccharides arise due to the formation of anomers (*i.e.* carbohydrate stereoisomers which differ only in the configuration at the hemiacetal carbon atom) and interconversion between pyranose and furanose rings.[150] Interconversion of the anomers occurs *via* the open chain form of the sugar, whilst mutarotation results from the opening and closing of the ring. Five tautomeric forms (*i.e.* structural isomers which are directly interconvertible) are usually obtained for each single sugar[151] – two pyranose, two furanose and the open chain form – and because they possess slightly different physical properties, they are generally separated by GC.[152] This interconversion can be minimised by the use of rapid and mild derivatising conditions. Where silylation is the chosen method of derivatisation, protection of the keto group of the monosaccharide prior to silylation precludes the formation of enol-TMS ethers, which are unstable and give rise to multiple products which cannot be prepared

quantitatively. Formation of the oxime derivative of the monosaccharide is the usual method of protecting its keto group.[148]

As already stated, sugars exist in both ring and straight chain forms but only the straight chain form possesses the keto group, which reacts with a pyridine solution of hydroxylamine hydrochloride to yield the oxime.[148]

The reaction by which the oxime is formed is that of nucleophilic substitution and the rate of formation is at a maximum under acidic conditions. The hydroxylamine hydrochloride is in equilibrium in the presence of pyridine:



The protonated form does not take part in the reaction - the presence of basic pyridine controls the equilibrium to the left by removing most of the acidic HCl from the mixture. The mechanism of the oxime formation is detailed in figure 6.5.

The trimethylsilylation of all the hydroxyl groups of the sugar-oxime intermediate completes the derivatisation of the sugar molecules, reducing the polarity of the molecules and therefore improving their chromatographic behaviour. If the polarity was not reduced, the compounds would be retained on the column and separation would not be achieved. Both the *Z* and *E* forms

of the oxime-TMS derivatives are produced due to the presence of the geometric isomers of the straight chain sugar molecules.

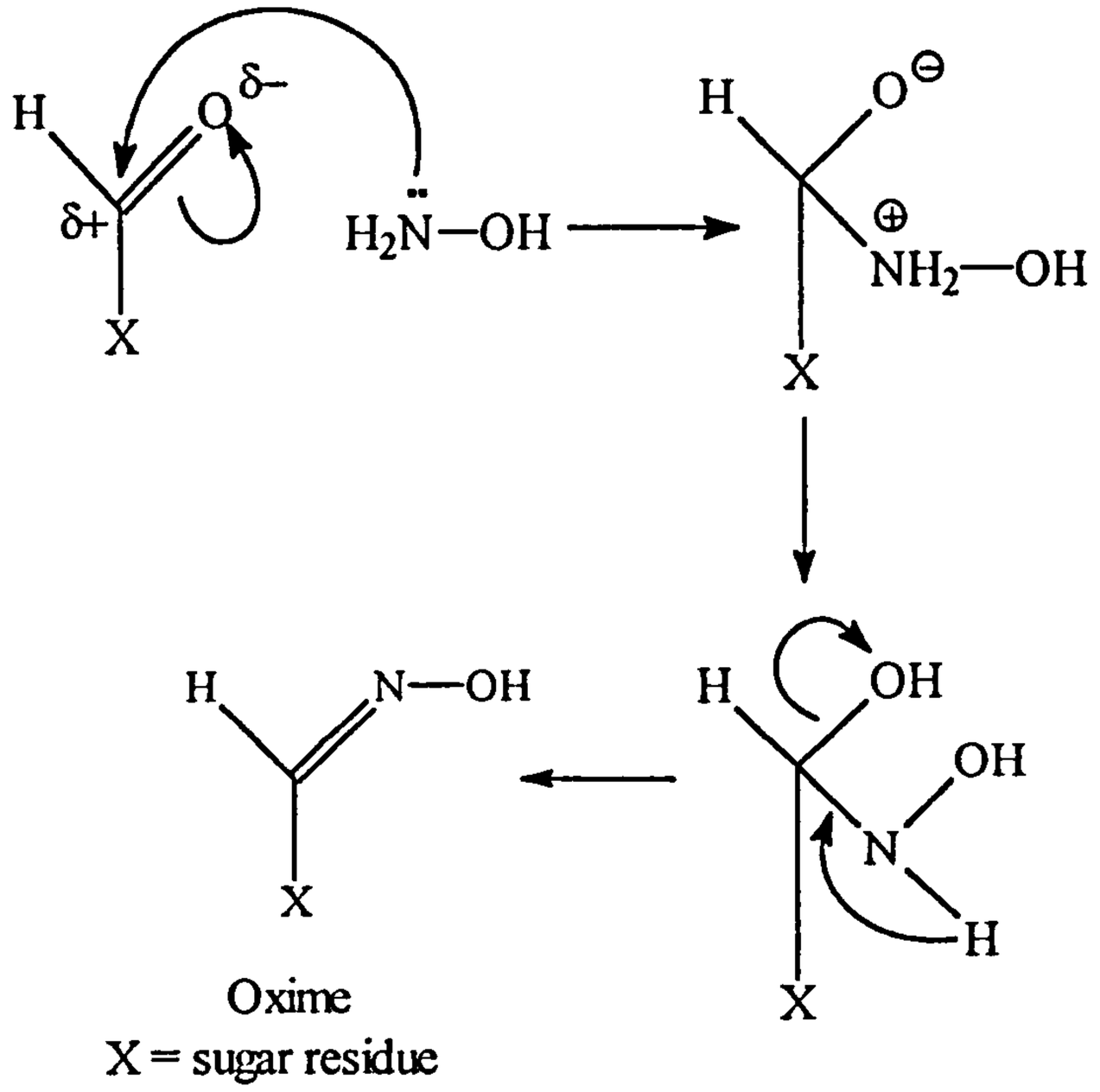


Figure 6.5: *Mechanism of Oxime Formation*

Despite the obvious advantages of the oxime method of sample preparation for the GC analysis of carbohydrate material, *i.e.* relatively uncomplicated chromatograms, it was decided that the oxime-TMS derivatives would not be utilised in this particular investigation. The primary problem with samples of gum material removed from works of art is the sample size: subnanogram levels of material are available for analysis and thus maximum concentration of the samples is paramount to aid their detection and subsequent identification. Reaction volumes should be kept to

a minimum, hence any additional procedures using reagents which are not absolutely necessary and which would ultimately cause further dilution of the sample should be avoided. Furthermore, the preparation of sugar oxime-TMS derivatives is more time-consuming than the preparation of TMS derivatives using HMDS, thereby increasing overall analysis times.

6.2.3 Derivatisation of Uronic Acids

There appears to be a lack of repeatability in the derivatisation of the uronic acid components present in some of the gum samples. It has been reported that the uronic acid linkage may be more resistant to acid hydrolysis than the glycoside linkage of the neutral monosaccharides.[153] The carboxylic acid function may result in the stabilisation of the linkage, thus yields of uronic acids after acid hydrolysis may be lower than expected.[154] The hydrolysed uronic acids become lactonised, the formation of the anhydrides occurring due to the intramolecular elimination of water between the hydroxyl and carboxyl groups of the uronic acid: the degree of lactonisation is not reproducible.[154,155] Methanolysis, an alternative technique which yields methyl glycosides, causes less degradation of the released sugar residues than acid hydrolysis and is equally efficient for the hydrolysis of neutral sugar glycoside linkages.[130,156,157]

6.2.4 Selected Ion Monitoring

A mass selective detector (MSD) provides the ideal detection technique in the GC analysis of natural sugars: the high sensitivity of the

MSD ensures that even the minute quantities of gums present in samples removed from works of art can be detected and characterised.

The first stage in a GC-MS analysis can be considered as a qualitative identification using a scan across the full mass range. The entire mass range is monitored over time, measuring the mass/charge ratios of ions within that range. The total response of the repeated scans forms the total ion count (TIC).

If a filter is selected to allow a smaller number of ions through to the detector the sensitivity is increased. Specific ions only are scanned over time, which means that more data per ion can be collected in a given period thus increasing the sensitivity. This technique is known as selected ion monitoring (SIM) and was utilised in the investigations into the characterisation of natural gums used as artists' binding media.

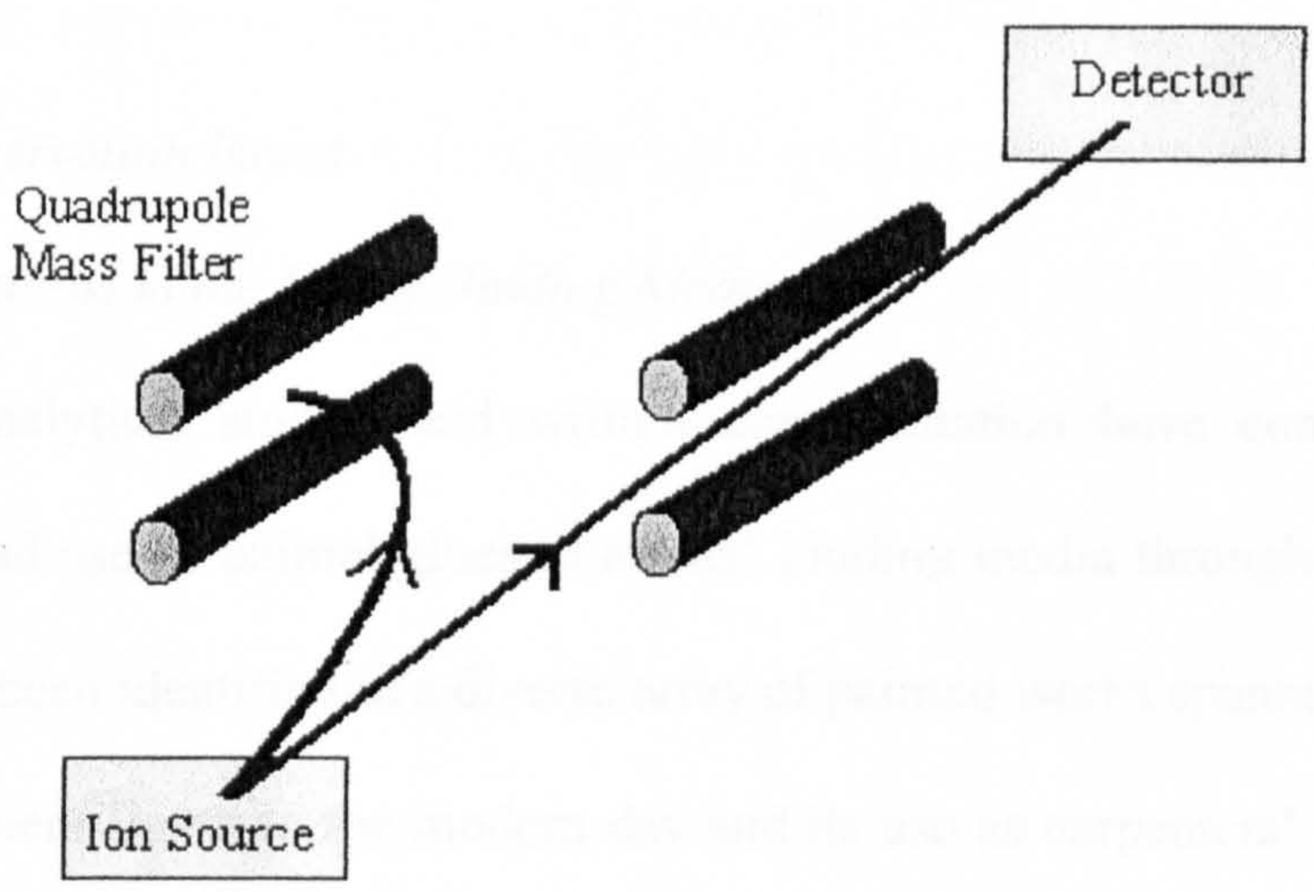


Figure 6.6: *A Quadrupole Mass Selective Detector*[158]

6.3 Conclusions I

It is clear that the methods developed in the course of these investigations are suitable for the analysis and characterisation of minute samples of proteinaceous and natural gum binding media removed from works of art.

RP-HPLC of amino acids in protein hydrolysates following derivatisation with FMOC provides a suitably sensitive and repeatable method for the identification of proteins commonly used by artists.

The qualitative GC-MS analysis of volatile sugar derivatives (silylation *via* HMDS) from natural gums provides a "fingerprint" which facilitates the characterisation of gum materials present in watercolours *et cetera*. The use of selected ion monitoring provides the additional sensitivity required for the detection of the almost trace levels of sugars present in these particular samples.

6.4 Conservation Issues

6.4.1 Patterns in the Use of Binding Media

Analytical studies and written documentation have confirmed the widespread use of animal glues in artists' binding media throughout history. Glue has been identified in a diverse array of painted works spanning the ages from Ancient Egypt to the modern day and its use as carpenters' adhesive is well known. Artists have frequently utilised animal glue as a size or sealant for canvas, paper, wood and stone supports prior to painting, a varnish coating and as a binder in gesso grounds and frame mouldings.[7] In addition, conservators have used glue for consolidation of flaking paint, retouching and

relining canvases.[55] Glue preparations for specific purposes have been described by many practitioners, including Pliny the Elder and Cennino Cennini in the 1st and 15th centuries respectively.[159,160]

In the 18th century du Monceau[161] advised the use of animal glues prepared from the hides of mature animals in cases where strength was of primary importance since he believed that "the older and leaner the animal, the stronger the glue". For painting, he preferred the glues from younger animals, since they were clearer and weaker.

Despite the widespread availability and use of animal glue media, it appears that artists were more inventive in their use of painting materials. The fact that animal glue is highly hygroscopic thus tends to flake with time and supports mould growth and insect attack probably led artists to explore other materials in the hope of achieving more durable water-based paint media.

The use of egg proteins and casein alone and as additives to animal glue media appears commonplace: the analysis of samples taken from works dating from the mid-sixteenth to the early twentieth century has revealed the widespread presence of these materials in primers and paints.

The use of gum mixtures in nineteenth century watercolour works has also been revealed in the course of these investigations. Gum arabic has long been acknowledged as the primary medium for watercolours, but is seems that this is not necessarily the case, with many paint samples containing gums tragacanth and karaya either in addition to or instead of gum arabic. This exciting and somewhat unexpected discovery will be discussed in detail in section 6.4.4.

Blake (1757-1827) did not favour oil media, claiming that colours were deadened by the oil which tends to darken and yellow with age. He experimented with different media, using watercolour and a method he referred to as "fresco", whereby his colours were ground in glue and applied over a priming of glue and whiting.

The analytical results of samples of paint media and priming taken from Blake's temperas show the overwhelming presence of animal glue in each work and reveal that he routinely added natural gums and cane sugar to his media, probably to inhibit cracking of the glue upon drying.

Priming from two works on canvas by Turner (1775-1851) was submitted for analysis: priming from the earlier work (*The Battle of Trafalgar...*) contained animal glue, whilst the later work (*Judith with the Head of Holofernes*) had a mixed egg albumin and casein priming. Turner also used this egg albumin and casein mixture as an adhesive in another work of a similar age. All of Turner's watercolours which were submitted for analysis in this investigation had paint layers which appear to comprise a mixture of gums tragacanth and karaya, similar to Blake's watercolour *The Simoniac Pope*, though the additional presence of cherry gum in the latter is suspected.

Like Turner and Blake, Edward Ward (1816-1879) used gum tragacanth in his early nineteenth century watercolour *Sketch for Gordale Scar*, whilst analysis of two of Rossetti's mid-nineteenth century works on paper revealed the use of more unusual media. It appears that Rossetti (1828-82) chose seed and plant mucilages more commonly found in Eastern art for his paint, though the exact nature of these substances remains unconfirmed to

date. Despite this use of unusual binding materials, Rossetti employed a more traditional animal glue and egg yolk mixture as an adhesive for his work *The Tune of the Seven Towers*, possibly because its properties were more predictable.

6.4.2 The Definition of Tempera

Throughout art history, the definition of "tempera" as a painting medium has changed many times, encompassing all paint media in its widest sense to egg media alone in its narrowest.[162]

Up until the 15th century, when painting with oil media became popular, the term "tempera" most likely referred to all media types, in the sense that pigments were tempered with the medium during paint formulation. However, once oil painting was established, the meaning of tempera was generally confined to paint media prepared from egg. Today, this would probably be described more accurately as "egg tempera".

The definition broadened, particularly during the nineteenth century, to incorporate any water soluble albuminous, gelatinous (*i.e.* derived from gelatine) and colloidal materials, such as natural gums:[162] specific terms, such as glue tempera for example, should be used to clearly define the media. In the late nineteenth century the term "distemper" was often substituted for "tempera" and used in the same context. If this particular definition of tempera is considered, virtually all pre-15th century painted works fall into the category of tempera paintings. Before the introduction of oil media, wax was the only non-aqueous binding medium available but after the late Middle

Ages, oil painting became the principal technique: despite this, tempera media did not become entirely obsolete.

Animal glue media were commonly used for panel paintings, illuminations and even wall paintings in India, China and Japan though the exact nature of the glue differed from place to place, making use of any available animal tissues suitable for the preparation of glue media.[162]

There is little evidence to suggest that the practice of painting with egg media stretched beyond European boundaries, with the exception of a medium prepared from salmon roe which was used by the native Indians of western Canada.[163] Certain sources claim that egg media prepared from both albumin and yolk were common in ancient Egypt, though the analysis of paint media removed from a tomb dating from the 29th Dynasty revealed the presence of natural gums. In 1934 Lucas stated that all Egyptian mural paintings had tempera media, though the exact nature of the tempera was not specified:[164] previously, in 1932, he had suggested that since domestic fowl were not native to Egypt but were in fact introduced to the country, the use of egg media was not likely in early works[165] – this indicates the use of the term "tempera" in the sense of any water soluble medium.

Virtually all the easel and wall paintings of classical Greece have disappeared from existence, along with the paint layers from classical Greek polychrome sculptures, though documentary evidence suggests that a variety of tempera media, including glue, egg and natural gums, were used by the artists/sculptors.[166] Reference was made to a particular mixture of egg and gum added to bile which made the colours flow easily.

A glue and egg albumin mixture was often employed in later times, as a medium for "mosaic gold" amongst other uses, though egg proteins were evidently the primary tempera media described by Cennini, the leading observer of Italian painting practices.[167] Other manuscripts of European origin[168] mention the use of mixed egg white and gum media along with some rather more unusual medieval mixtures for tempera paints, including fish glue with lime, ashes, wax, and mastic[169] and a mixture prepared from flesh boiled in water with plant roots.[170] The use of natural gums as tempera is also described in the Strasbourg manuscript, dating from the 15th century.[171]

However, the major tempera medium of European art from the Middle Ages and early Renaissance was undoubtedly egg, either whole or as egg white or yolk: Cennini himself recommended the use of egg yolk alone saying "...you must always temper your colours with yolk of egg, and get them tempered thoroughly – always as much yolk as the colour you are tempering".[172] Despite this advice it is unlikely that the use of egg tempera remained in general painting practice beyond the 16th century, whilst the gum temperas used in inks for writing, drawing and illuminating developed into the medium we know today as watercolour.

As stated previously Blake, one of the greatest exponents of tempera painting, did not favour the use of oils in his works claiming that "Oil will not drink or absorb colour enough to stand the test of very little time and of the air; it grows yellow, and at length brown. It was never generally used till after Vandyke's time. All the little old pictures, called cabinet pictures, are in fresco, and not in oil".[173]

Blake preferred to use a technique he called "fresco", which he claimed would be "unchangeable and permanent": his biographer Gilchrist described his fresco technique as involving the application of colours ground in "common carpenters' glue" to a very thick priming of glue and whiting on canvas, wood or metal.[174] Today Blake's technique is classified as tempera.

Blake was so enthusiastic about the merits of tempera painting that in 1809 he arranged an exhibition of his works at his brother's house. The exhibition featured nine of his temperas and seven ordinary watercolours, but to his disappointment attracted little attention. In direct contrast to Blake's own feelings, his temperas have remained overshadowed by his watercolours and drawings, never being fully appreciated as the masterpieces they were clearly intended to be. This is largely due to the fact that Blake's claims of permanence have proved unfounded over time and today those temperas which have survived have deteriorated to a great extent, whilst his watercolours remain as vibrant and stimulating as in Blake's day.

6.4.3 Effects of Binding Media and Support on Condition of Works

The condition of ageing works is clearly dependent on the type of priming, binding media and support chosen by the artist. Consider Blake's temperas between 1799 and 1800. Blake's use of tempera in preference to oil has proved regrettable in that most of his works in the medium have decayed to such an extent that, at best, they bear no resemblance to their originally intended appearance – many of the larger tempera works have not even survived into the twentieth century.

The temperas analysed in this study are primarily on stretched canvas supports, though one is on a tinned steel plate with a layer of red lead in oil to prevent corrosion. The works can be separated into two groups with respect to their physical condition, though sadly all are deteriorating – those displaying a reasonable level of flaking/cracking and those with extensive flaking/cracking.

It can be seen that those temperas which have extensive flaking/cracking of the paint layers, *i.e. The Flight into Egypt, Christ the Mediator* and *The Agony in the Garden*, contain animal glue alone in the priming and paint. The severe deterioration of these temperas is undoubtedly the result of the thickness of the layers: Blake was inclined to apply his priming of glue and whiting very thickly, almost as if he was plastering a wall. The priming itself contains only a small amount of actual binding medium and is extremely brittle. Consequently, when applied to a flexible and moisture sensitive canvas support cracking is inevitable. Animal glue reacts to moisture much faster and more severely than other proteinaceous medium, changing in its dimensions and therefore putting stress on the other materials in the paint or priming layers. The actual structure of the paint layers themselves exacerbates this problem of flexibility and moisture sensitivity– the use of thick layers of animal glue on either side of the actual pigmented layer increases the inflexibility of the paint. Although *The Agony in the Garden* has a less flexible steel plate support, the extreme thickness of the glue priming has ultimately resulted in extensive cracking of the paint layers: all thick applications of glue will crack eventually, whether the support is flexible or not.

The Body of Christ Borne to the Tomb and *Bathsheba at the Bath*, appear to be in slightly better condition, displaying less flaking/cracking of the surface. This is probably due to the presence of gum material and cane sugar in the priming and paint layers, which act as plasticisers and thus assist in the retention of flexibility. As mentioned previously, Blake probably added these ingredients to prevent the thick glue layers cracking upon drying.

The Spiritual Form of Nelson Guiding Leviathan and *The Bard, from Gray* date from 1805-9, though Blake's technique undoubtedly remained largely unchanged. Samples of priming and paint media removed from both works were submitted for gum analysis only, but staining tests performed on cross-sections by the Tate Gallery's Conservation Department revealed the presence of animal glue in a layered structure. The paintings are badly cracked, probably due to the use of very thick layers of glue as illustrated previously.

The final tempera analysed, *The Ghost of a Flea*, is painted on an inflexible hardwood support and is thus comparable in structure to *The Agony in the Garden*. The work contains mixed glue and gum media in the priming and paint and is extensively cracked, almost certainly as a result of the extreme thickness of the glue layers applied by Blake.

Blake's two watercolours *The Simoniac Pope* and *The Blasphemer*, thinly painted on paper supports sized with glue, are the only works of those studied which remain undamaged. The paint medium of *The Simoniac Pope* has been identified as a gum mixture, probably containing gums cherry, tragacanth and karaya. Contamination prevented the analysis of samples from *The Blasphemer*, though it is safe to assume that the paint medium is

likely to be the same in both works, given the similarity in their appearance. It is unquestionably the absence of any priming (minimising the overall thickness of the works) and a thick application of animal glue which has spared these two watercolours from the fate met by the temperas. An aqueous watercolour medium retains its flexibility on drying and is thus less prone to cracking.

Watercolour works by Turner and E.M. Ward from the first half of the 19th century are also in good condition, showing little or no flaking or cracking of the paint surface – each work is painted on an unprimed paper support sized with glue. The three works by Turner, *The Bridge and Goats, Mont Pilatus from Lake Lucerne*, and *Lake of Lucerne, looking from Kussnacht towards the Bernese Alps; Mont Pilatus on the Right, Dark against the Sunset* and Ward's *Sketch for Gordale Scar* all contain only gum paint media. The artists did not use animal glue as a paint medium and since paint layers are very thin in watercolours, maximum flexibility is ensured thus reducing the propensity of the work to crack and flake.

Comparisons can be drawn between Turner's work *The Battle of Trafalgar...* and Reynold's painting *Sir James Hodges* – both are painted on canvas and have been glue lined at least once. An egg priming medium was expected for Turner's work, but on analysis was found to be pure animal glue: it is highly likely that the animal glue from the heavy lining has penetrated the priming over time, thus giving the impression that the priming medium is in fact glue. The penetration of glue from the lining leads to a gradient of glue through the priming into the paint layers themselves, the extent of penetration is determined by the pigment to medium ratio.

Date	Work	Condition	Support	Priming	Paint
1799	BLAKE *The Flight into Egypt*	Extensive flaking/cracking	Canvas	Typically glue/whiting	Animal glue
1799-1800	BLAKE *Christ the Mediator*	Extensive flaking/cracking	Canvas	Typically glue/whiting	Animal glue
	BLAKE *The Agony in the Garden*	Extensive flaking/cracking	Steel plate	Animal Glue	Animal glue, karaya gum, gum tragacanth mixture
	BLAKE *Body of Christ Borne to the Tomb*	Reasonable flaking/cracking	Canvas	Karaya gum, and cane sugar mixture	Animal glue only and mixture of animal glue, gums tragacanth, arabic and cane sugar.
	BLAKE *Bathsheba at the Bath*	Reasonable flaking/cracking	Canvas	Animal glue, gums arabic, tragacanth and cane sugar mixture	Animal glue, gums arabic, tragacanth and cane sugar mixture
	BLAKE *The Blasphemer*	No flaking/cracking	Paper	No priming	Assumed to be same as *The Simoniac Pope*
1805-1815	BLAKE *Spiritual Form of Nelson Guiding Leviathan*	Extensive flaking/cracking	Canvas	Gums karaya, tragacanth and cane sugar mixture Stained for animal glue	Gums karaya, tragacanth and cane sugar mixture
	BLAKE *The Bard, from Gray*	Reasonable flaking/cracking	Canvas	Typically glue/whiting Stained for animal glue	Gums karaya, tragacanth and cane sugar mixture
	TURNER *The Bridge and Goats*	A little flaking/cracking	Paper	No priming	Gums karaya and tragacanth mixture
	E.M. WARD *Sketch for Gordale Scar*	No flaking/cracking	Paper	No priming	Gum tragacanth

Table 6.1: *Priming and Paint Media from 18th and 19th Century Works*

Date	Work	Condition	Support	Priming	Paint
1816-1825	BLAKE *The Ghost of a Flea*	Extensive flaking/cracking	Wooden panel	Animal glue and gum arabic mixture	Animal glue, gums arabic, tragacanth, karaya and cane sugar mixture
	BLAKE *The Simoniac Pope*	No flaking/cracking	Paper	No priming	Gums karaya, tragacanth, cherry mixture
1845	TURNER *Mont Pilatus from Lake Lucerne*	No flaking/cracking	Paper	No priming	Gums tragacanth and karaya mixture
	TURNER *Lake of Lucerne.....*	No flaking/cracking	Paper	No priming	Gums tragacanth and karaya mixture
1855-1865	ROSSETTI *Dr Johnson at the Mitre*	A little flaking/cracking. Some blooming	Paper	No priming	Tamarind seed mucilage, possibly
	ROSSETTI *How Sir Galahad.....*	Moderate flaking/cracking overall, but extensive in areas	Paper	No priming	Brown seaweed mucilage, possibly
1922	E. WADSWORTH *Near Marseilles*	A little flaking/cracking	Canvas	Unknown	Egg albumin and animal glue mixture

Table 6.2: *Priming and Paint Media from 19th and 20th Century Works*

Date	Work	Condition	Support	Priming
1550	Follower of HOLBEIN *William, First Lord de la Warr*	A little flaking/cracking	Hardwood panel	Animal glue and casein mixture
1570-1580	BRITISH SCHOOL *An Allegory of Life*	Some flaking/cracking	Oak panel	Animal glue
	KETEL *Alice Smythe, Robert Smythe and Joan Smythe*	A little flaking/cracking	Hardwood panels	Animal glue and egg yolk mixture
1765	REYNOLDS *Sir James Hodges*	A little flaking/cracking	Canvas	Animal glue, egg and casein mixture
1805-1830	TURNER *The Battle of Trafalgar...*	No flaking/cracking	Canvas	Animal glue
	TURNER *Judith with the Head of Holofernes*	Moderate flaking/cracking	Canvas	Egg albumin and casein mixture
1913	D. GRANT *The Ass*	A little flaking/cracking	Paper	Casein
1985	J. LESSORE *Apollo and Daphne*	No flaking/cracking	Canvas	Animal glue

Table 6.3: *Priming Media from Miscellaneous Works (Aged and Modern)*

Date	Work	Condition	Support	Adhesive	Coating
1822	TURNER *George IV's Departure from the 'Royal George'*	Adhering well	Paper and hardwood panel	Casein and egg albumin mixture	*****
1857	ROSSETTI *The Tune of the Seven Towers*	Adhering well	Paper	Animal glue and egg yolk mixture	*****
1895	FEHR *The Rescue of Andromeda*	A little flaking/cracking	Bronze	*****	Animal glue and egg yolk in wax-based mixture

Table 6.4: *Adhesives and Coatings from 19th Century Works*

However, the presence of this absorbed glue in the priming still affects the condition of the work in the same way as if it had been the original medium. *The Battle of Trafalgar*... has little flaking or cracking of the paint and since Turner did not tend to use the thick layers of glue priming favoured by Blake it is probably this less heavy-handed approach which has better preserved his works.

Sir James Hodges slightly pre-dates Blake's temperas. Animal glue was again detected in the priming, in addition to other proteinaceous materials, but this is probably due to penetration of glue from the lining into the priming as mentioned previously. The work, painted on a flexible canvas support, has very little damage which adds further credence to the proposed theory regarding the influence of animal glue on aged paintings.

Consequently, it is clear that the mere presence of animal glue itself does not necessarily lead to deterioration of the paint layers, rather that it is the excessive application of glue which ultimately results in extensive cracking. Evidence provided by the analysis of media samples removed from much older works, dating as far back as the sixteenth century, appears to confirm this.

The most conclusive evidence is provided by the analysis of priming samples from *William, First Lord de la Warr* (follower of Holbein), *An Allegory of Life* and the three portraits by Ketel: all contain animal glue, either alone or as part of a mixed medium, yet each work displays only a small amount of flaking or cracking. The works are all painted on inflexible hardwood panels but if just the presence of animal glue was sufficient to cause severe deterioration, surely these 400 year old paintings would be in

very poor condition indeed. The fact that they remain relatively undamaged indicates that it is the way in which the animal glue medium is applied by the artist that is the most important influence on the condition of aged works of art.

Tables 6.1 to 6.4 summarise the analytical results obtained for each sample removed from the works of art under investigation: additional details regarding the nature of the support and the condition of the works are also given.

6.4.4 Additives to Gum Media

It has always been assumed that artists used predominantly gum arabic for their watercolours, or when a gum material was added as a modifier to some other medium, such as animal glue. However, the results obtained from these investigations into the nature of artists' binding media appear to cast doubts as to the accuracy of this generalisation.

Many of the works from the Tate's collection which were submitted for analysis contained natural gums as binding materials, but the diversity of gums actually found is surprising.

It is likely that artists consciously added cane sugar or honey to paints as a plasticizer or humectant to reduce the cracking of dried paint films: Blake often used cane sugar since his typically thick layers of paint required an increase in their natural flexibility. Turner did not add sugar to his paints, probably since he painted more thinly, though in areas where he used a thicker gum wash there is obvious cracking.

Less commonly used gums, including tragacanth, karaya and cherry, were detected in addition to gum arabic. Each of Turner's watercolours contains a mixture of gums tragacanth and karaya, rather than gum arabic, whilst Blake's works contain complex mixtures of gums arabic, tragacanth, karaya, cherry and cane sugar: a previous analysis by thin layer chromatography of gum media removed from another of Blake's watercolours revealed the probable presence of gum karaya.[175] Ward's *Sketch for Gordale Scar* appears to contain only gum tragacanth, previously considered to be used primarily in the manufacture of artists' pastels; however, its use as a painting medium did become more common from the late nineteenth century.

Chromatographic evidence also suggests the possible use of seaweed and tamarind seed mucilages, known to have been popular in Eastern art. The use of such materials is little documented in Western art, though painters of Rossetti's era were known to be influenced by Eastern art: information on other painting techniques and materials was more freely available, thus experimentation with unusual media was highly likely.

The results obtained from the analysis of samples removed from works of art from the late eighteenth and nineteenth centuries suggests two possibilities: firstly, artists were far more creative with their selection of gum media (in the same way that they experimented with proteinaceous media), consciously choosing to mix unusual, possibly more expensive[20] gums with gum arabic or, secondly, unscrupulous importers or dealers adulterated less readily available gums with gum arabic for a variety of reasons, *e.g.* to

increase profits or where the availability of supply was unpredictable. This interesting hypothesis may itself be worthy of further historical investigation.

6.5 Conclusions II

These investigations have proved the feasibility of RP-HPLC and GC-MS methods for the characterisation of proteinaceous and natural gum materials used by artists as binding media.

It has become clear that the way in which media are used influences the condition of aged works more than the type of medium alone: the use of thick layers of animal glue appears to cause maximum deterioration in paintings, especially when it is applied to flexible and moisture-sensitive supports such as canvas.

Furthermore, it seems that artists were more inventive in their choice of materials than previously thought, selecting many rare natural products for their paints – experimentation was necessary in order to improve paint durability and discover methods of achieving special effects.

However, in order to add further credibility to these postulations, it is necessary to study a wider range of samples, *i.e.* samples removed from works by many different artists from different geographical locations and periods.

PROSPECTS FOR FUTURE WORK

Future work can be divided into two categories, that related to experimental methodology and that related to the investigation of artists' materials and techniques. Consider first the work to further develop the experimental methodology.

The analysis of proteinaceous media samples was achieved *via* RP-HPLC, after acid hydrolysis and derivatisation of the samples. Hydrolysis was performed using hydrochloric acid in its liquid form, which meant that samples required buffering to around pH 7 prior to their derivatisation. However, if vacuum hydrolysis was used instead, *i.e.* with constant boiling hydrochloric acid under vacuum conditions, there would be no need to buffer the samples, the overall reaction volume would be reduced thus increasing sample concentration, hence sensitivity would effectively be increased. A simple gas-phase procedure for the hydrolysis of proteins has been reported[176] and an investigation into the suitability of this procedure for the hydrolysis of proteinaceous artists' media would be desirable.

Furthermore, if sample volumes could be reduced by removing the need for buffering, a smaller volume of derivatising agent would be required: if the volume of FMOC solution was reduced to around 50 μl interference from hydrolysis products and the reagent itself would be minimal,[177] resulting in the eradication of the extraction procedure and ultimately minimising analyte losses during sample preparation.

Staining tests are routinely used to confirm the presence of an oil or proteinaceous medium[23,29,30] but a stain specific for gum media is still in a

developmental stage:[178] the use of a gum-specific stain would prove useful for revealing the actual structure of paint layers, showing the exact location of gum materials in relation to any oil or proteinaceous media present in the cross-section. Initial attempts to stain gum layers in cross-sections taken from some of Blake's works have proved successful.[178,179]

The use of ion-pair reagents is an option worthy of investigation, since it would remove the need for pre-column derivatisation of proteinaceous media samples. Ion-pair chromatography has been employed for the determination of free amino acids in solution[180] and could provide a useful method for the analysis of proteinaceous media hydrolysates, though a post-column derivatising agent suitable for both primary and secondary amino acids would be required.

Capillary zone electrophoresis (CZE) is another technique used for the analysis of proteins *et cetera*: the development and application of methods for the analysis of proteinaceous media samples could prove worthy of investigation

The full quantitation of the GC method for the analysis of gum media is one of the most important objectives to achieve. The development of a fully quantitative method would facilitate the use of discriminant function analysis in the characterisation of gum media, in the same way that the technique was utilised for the identification of proteinaceous media (see section 5.4.1). A more extensive investigation into the suitability of various silylating agents would also be useful.

As mentioned previously in section 6.2.3, the derivatisation of uronic acids present in the gum materials appeared to lack repeatability. A number

of methods for the simultaneous derivatisation and GC analysis of neutral sugars, uronic and organic acids have been reported.[78,181] Methanolysis provides an alternative to the acid hydrolysis of samples, resulting in less degradation of released sugar residues and efficient hydrolysis of the uronic acid glycoside linkage. The application of this technique to samples of gum media removed from works of art should be studied.

GC methods for the analysis of carbohydrate compounds are well known, but the HPLC analysis of dabsylhydrazine derivatives of monosaccharides in fruit juices and clinical samples has been reported.[182] Separation was achieved on a *Spherisorb* C18 reversed phase column with a gradient programme of water/acetonitrile eluent and UV detection, which means that the instrumentation currently employed for the analysis of proteinaceous media would also be suitable for investigations into gum media.

The second area of work concentrates on further investigations into artists' techniques and the materials available to them. In order to precisely determine any patterns evident in the selection of media by artists, it is necessary to study samples from a much wider range of works, *i.e* works from different artists, regions, periods *et cetera*. The analysis of a much larger number of samples should reveal how geographical location influenced the availability of materials, how particular materials became fashionable and if artists preferred to use only a limited range of materials throughout their careers.

Blake himself painted many more works than those investigated here and if a completely accurate account of his materials and techniques is to be

compiled, samples from a greater number of his paintings should be analysed. Comparisons between the materials used by Blake and his followers, *e.g.* Palmer, may also prove interesting – did he actually reveal his methods to his followers, or were they able to produce only their own interpretations of his technique?

The detection of gums other than arabic in such a limited number of samples has revealed the need for further investigations into the nature of binding media used in a larger selection of paintings and watercolours. Extensive studies of the materials used by Blake's contemporaries may provide further evidence in support of the theory that gums were adulterated by importers and dealers: indeed, examination of historical documents may indicate that gums were mixed to compensate for fluctuating supplies.

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APPENDIX 1

Glossary of Conservation Terms

Aqueous Media: Water-based media comprising materials which are water-soluble, *e.g.* proteinaceous materials such as animal glue, casein, egg, and natural gums such as gum arabic. Paints containing water-soluble gum media are generally referred to as watercolours.

Bloom: The cloudy, white appearance of the surface of aged varnish or paint, caused by minute cracks or pores in the surface, which diffuse light, or by the presence of low molecular weight components which have diffused to the surface. The term covers a variety of surface alterations.

Drying Oils: Oils which dry by oxidation, forming a solid yet elastic surface. Chiefly used in paints and varnishes, *e.g.* linseed oil, walnut oil, poppy oil and safflower oil.

Egg Tempera: Paint media comprising whole egg, egg yolk or egg white. Egg tempera prepared from egg yolk is traditional, though there are many different preparations for egg tempera media.

Extender, Filler: A colourless or white, usually transparent, inert material used to dilute or diffuse coloured pigments. When used in certain proportions, an extender can improve the durability of paints. The term

'filler' is sometimes used synonymously with extender, though technically a filler is a white, transparent, inert material with a low reflective index which is used in paste form to fill imperfections in the surface of the support.

Gouache: A watercolour paint rendered opaque by the addition of white pigments. It is applied to a paper support, though more thickly than traditional transparent watercolours.

Ground: The layers in a painting which lie between the support and the paint itself, whether supplied commercially or applied by the artist.

Medium: The term usually applied to the vehicle which binds together the pigment particles in paints, *e.g.* oil, animal glue, egg *et cetera.*

Non-Drying Oils: Vegetable oils which merely thicken when exposed to the air – they do not form a skin through oxidation, even after prolonged exposure.

Paint Film: A continuous and usually thin layer of pigment, extenders/fillers and medium combined.

Plasticisers: Non-volatile liquids or solids incorporated into lacquers or varnishes to ensure that the film retains its flexibility and elasticity after drying. Honey, sugar solution and glycerine have been used as plasticisers in water-based paint films.

Priming: The thin, continuous layer between the ground and the paint film, often confused with the 'ground' itself. However, this is not a universal definition and the Tate Gallery describes it slightly differently: if an artists purchased a support with a ground he may have applied a further layer himself prior to painting on the support – this layer would be called the priming. Generally priming includes pigment and filler, in addition to the medium itself; if there is no pigment or filler, it is a 'size'.

Resins: Natural resins are the secretions of certain plants, usually living, though copal and amber are from dead and fossilised plant sources. The resin may exude naturally, but is usually collected by wounding the tree with small incisions. Resins alter in consistency and colour on exposure to the air and light. Synthetic resins are complex organic solids or semi-solids specifically prepared to closely physically resemble natural resins. Solvent 'solutions' of natural and/or synthetic resins are used as varnishes.

Size: Any material used to prepare or fill a porous surface, *e.g.* glue size which is often used to prepare wooden surfaces for painting, or to render the ground layer non-absorbant.

Support: The term describing the physical structure carrying the ground and paint layers of a painting, *e.g.* canvas, panels, paper, walls *et cetera*.

Temper: To prepare a paint or make a material brushable; to blend or mix in proper proportions.

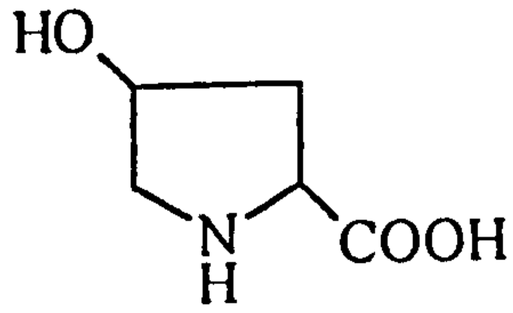
Tempera: Generally accepted as the term describing any aqueous albuminous, gelatinous or colloidal materials used as binding media, *e.g.* animal glues, egg proteins, casein and natural gums: specific terms such as glue tempera, egg tempera or gum tempera should be used to accurately define a particular medium.

Varnish: The coating, usually containing some form of resin, applied over the surface of a painting. It has two functions – to saturate the colours and to provide physical protection.

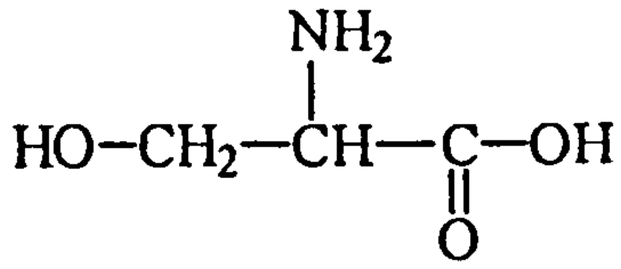
Watercolour: The accepted term for a standard preparation of pigment ground in a water-soluble gum, *e.g.* gum arabic, typically applied in thin, transparent layers to a paper support.

APPENDIX 2

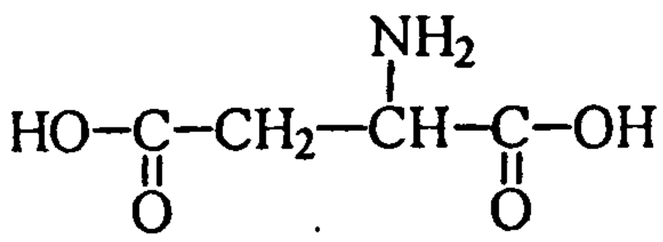
Structures of Underivatised Standard Amino Acids and Monosaccharides



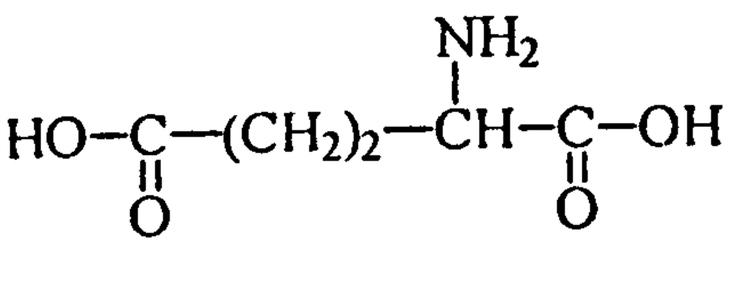
Hydroxyproline (OH-Pro)



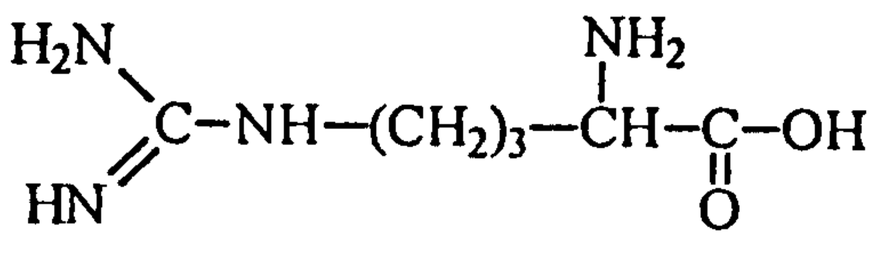
Serine (Ser)



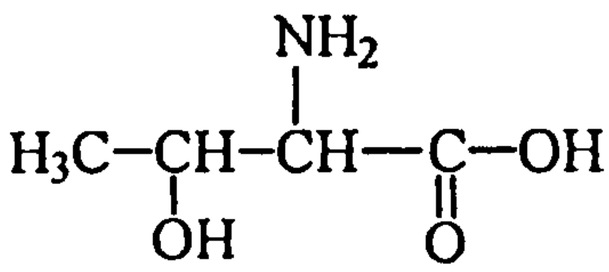
Aspartic Acid (Asx)



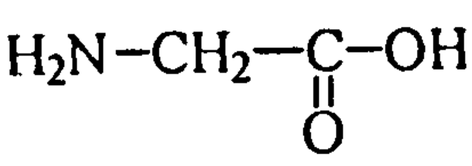
Glutamic Acid (Glx)



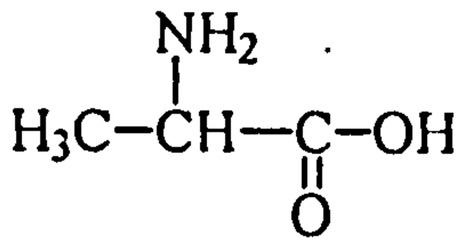
Arginine (Arg)



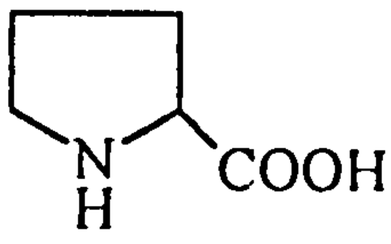
Threonine (Thr)



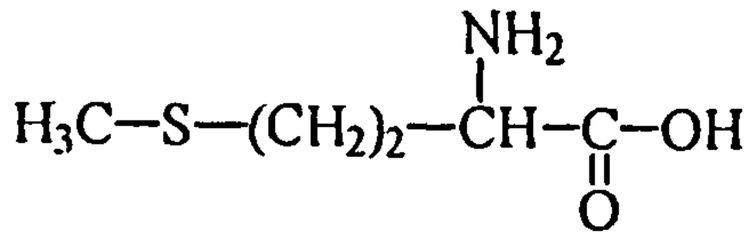
Glycine (Gly)



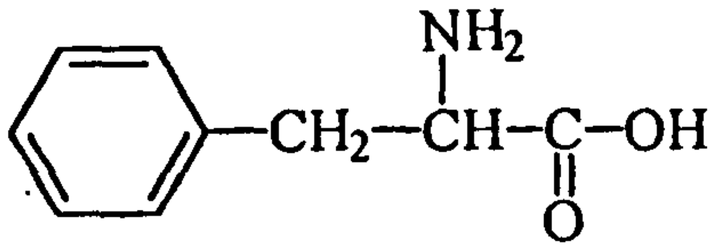
Alanine (Ala)



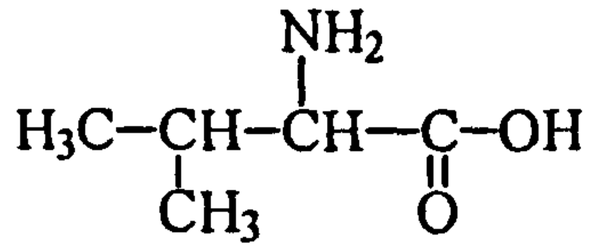
Proline (Pro)



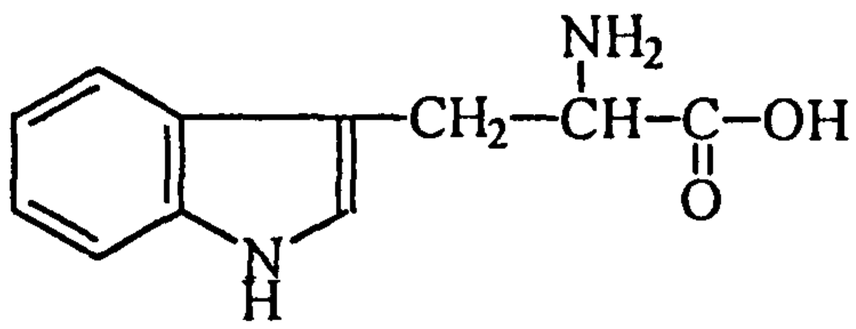
Methionine (Met)



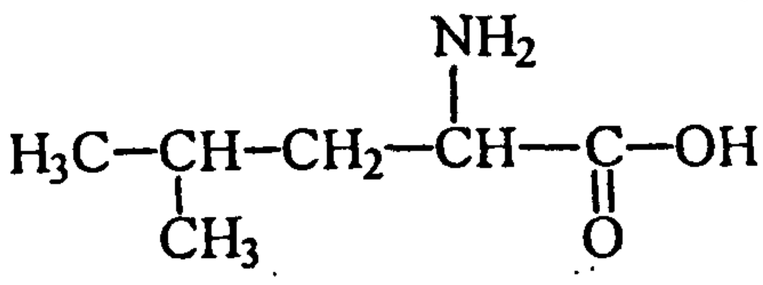
Phenylalanine (Phe)



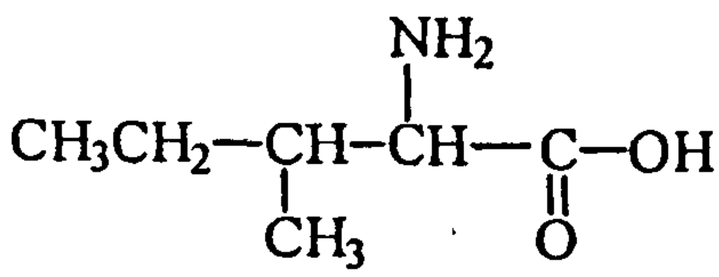
Valine (Val)



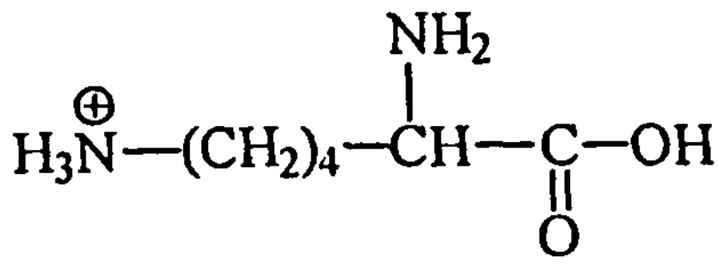
Tryptophan (Trp)



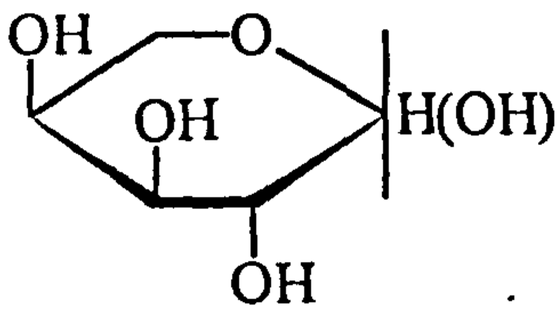
Leucine (Leu)



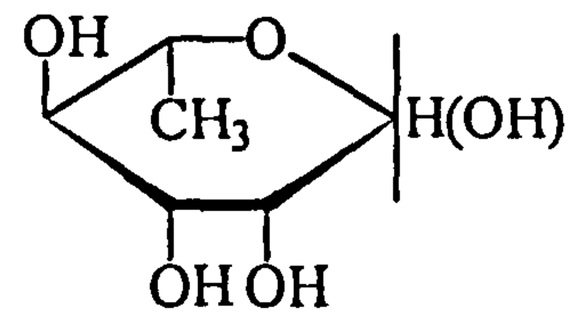
Isoleucine (Ile)



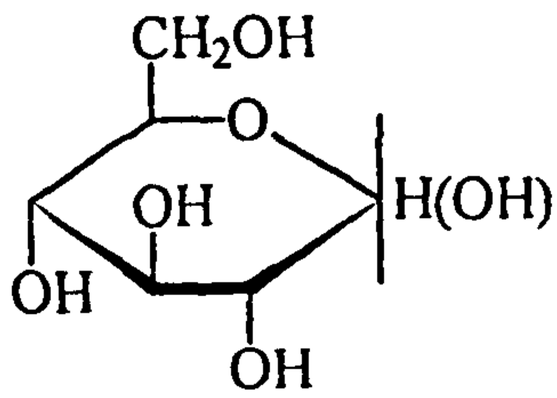
Lysine (Lys)



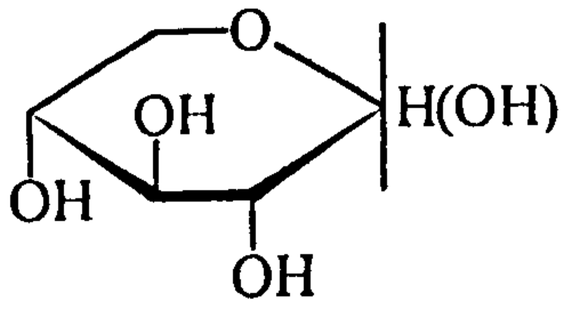
Arabinose



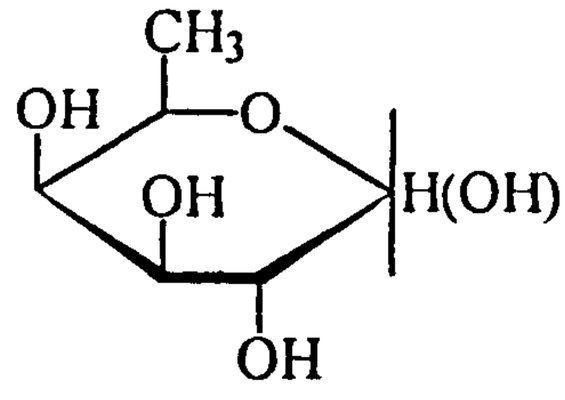
Rhamnose



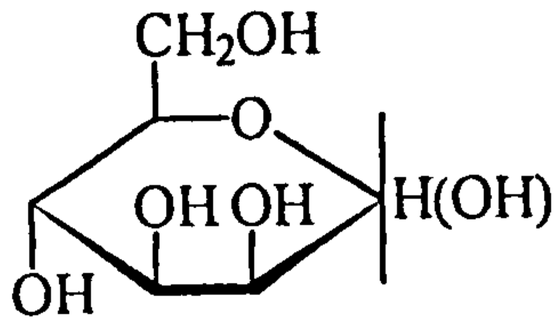
Glucose



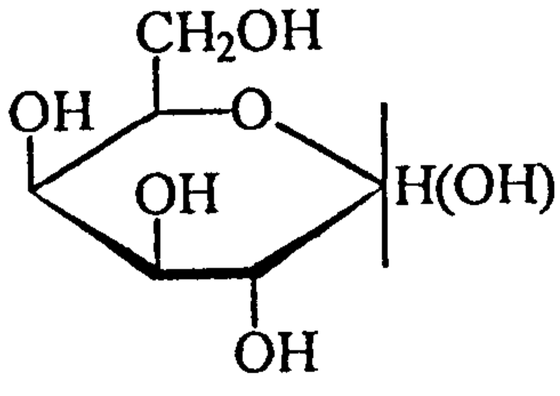
Xylose



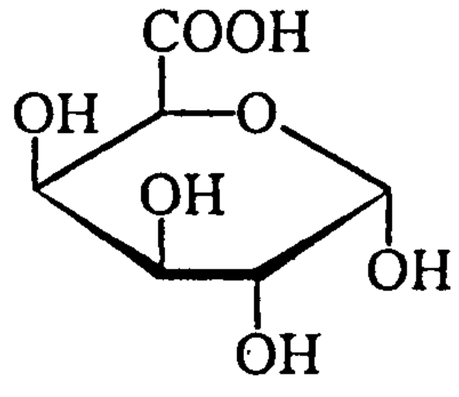
Fucose



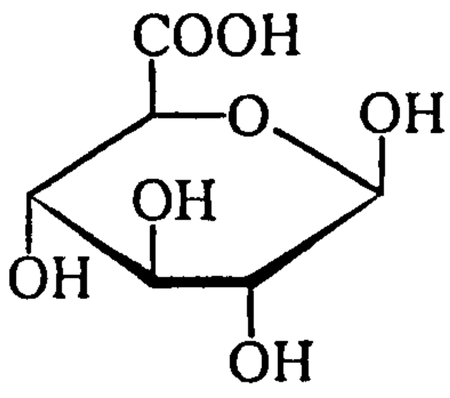
Mannose



Galactose



Galacturonic Acid



Glucuronic Acid

APPENDIX 3

Black and White Plates of Works by Blake, Turner and Rossetti

PLATE 1: BLAKE, William (1757-1827), *The Body of Christ Borne to the Tomb* (N01164), circa 1799-1800. Tempera on canvas, mounted on cardboard.

PLATE 2: BLAKE, William (1757-1827), *Bathsheba at the Bath* (N03007), circa 1799-1800. Tempera on canvas.

PLATE 3: BLAKE, William (1757-1827), *The Ghost of a Flea* (N05889), circa 1819-20. Tempera heightened with gold on mahogany.

PLATE 4: BLAKE, William (1757-1827), *The Agony in the Garden* (N05894), circa 1799-1800. Tempera on iron.

PLATE 5: BLAKE, William (1757-1827), *The Spiritual Form of Nelson Guiding Leviathan* (N03006), circa 1805-9. Tempera on canvas.

PLATE 6: BLAKE, William (1757-1827), *The Bard, from Gray*, circa 1809. Tempera heightened with gold on canvas.

PLATE 7: BLAKE, William (1757-1827), *The Blasphemer*, circa 1800. Pen and ink and watercolour on paper.

PLATE 8: BLAKE, William (1757-1827), Illustration to Dante's Divine Comedy, *The Simoniac Pope*, 1824-7. Pen and ink and watercolour on paper.

PLATE 9: TURNER, Joseph Mallord William (1775-1851), *The Battle of Trafalgar, as Seen from the Mizen Starboard Shrouds of the Victory* (N00480), 1806-8. Oil on canvas.

PLATE 10: TURNER, Joseph Mallord William (1775-1851), *George IV's Departure from the 'Royal George', 1822* (N02880), circa 1822. Oil on wood.

PLATE 11: TURNER, Joseph Mallord William (1775-1851), *The Procuress; Judith with the Head of Holofernes* (N05500), circa 1828. Oil on canvas.

PLATE 12: TURNER, Joseph Mallord William (1775-1851), *The Bridge and Goats* (D08147), circa 1806-7. Watercolour on paper.

PLATE 13: TURNER, Joseph Mallord William (1775-1851), *Mont Pilatus from Lake Lucerne* (D33496), circa 1845. Watercolour and gouache on paper.

PLATE 14: TURNER, Joseph Mallord William (1775-1851), *Lake of Lucerne, looking from Kussnacht towards the Bernese Apls; Mont Pilatus on the Right, Dark against the Sunset* (D33499), circa 1845. Watercolour and gouache on paper.

PLATE 15: ROSSETTI, Dante Gabriel (1828-1882), *The Tune of the Seven Towers* (N03059), 1857. Watercolour on paper.

PLATE 16: ROSSETTI, Dante Gabriel (1828-1882), *Dr. Johnson at the Mitre* (N03827), 1860. Watercolour on paper.

PLATE 17: ROSSETTI, Dante Gabriel (1828-1882), *How Sir Galahad, Sir Bors and Sir Percival Were Fed with the Sanct Grael; but Sir Percival's Sister Died by the Way* (N05234), 1864. Watercolour on paper.

PLATE 1

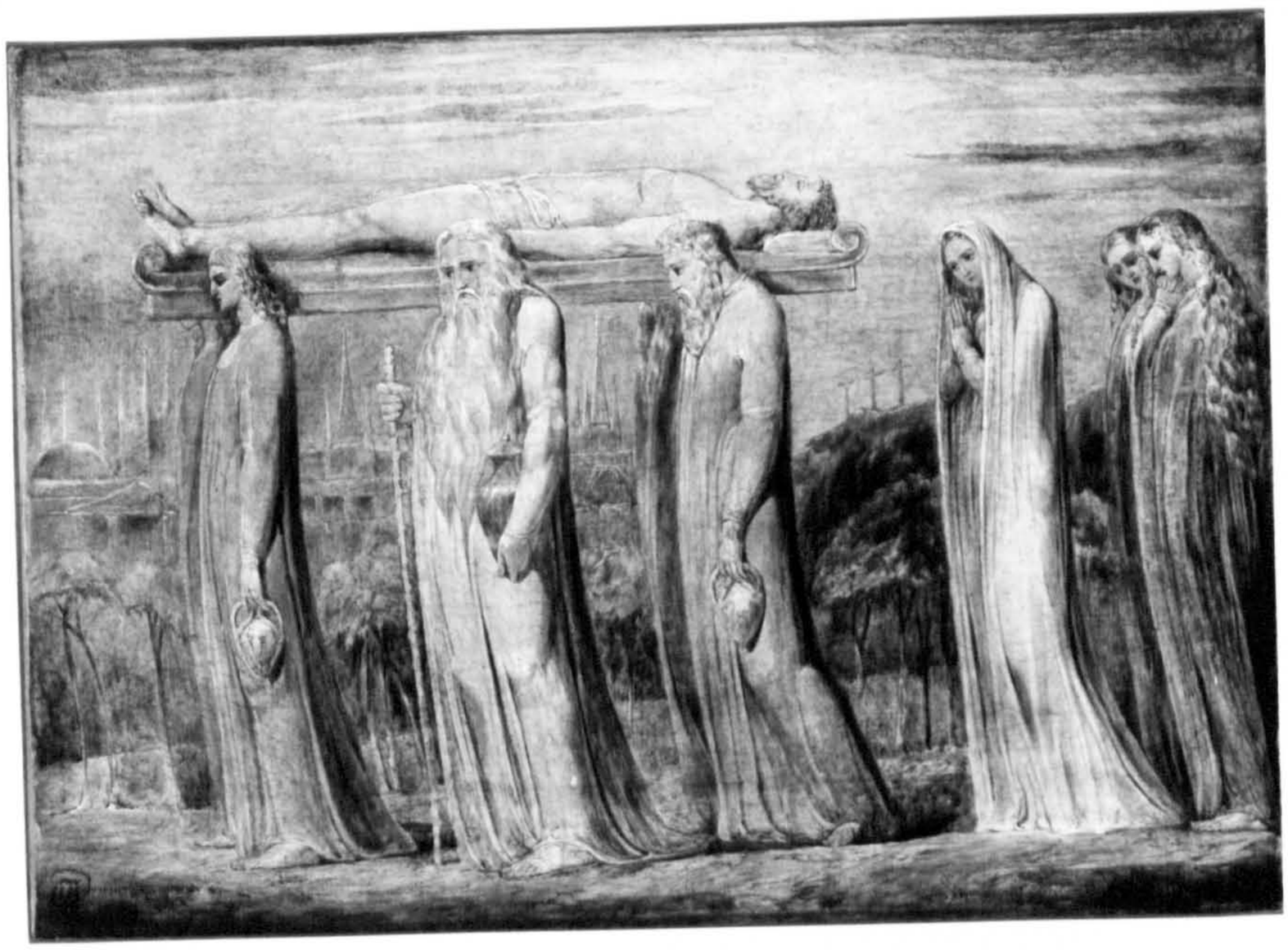


PLATE 2

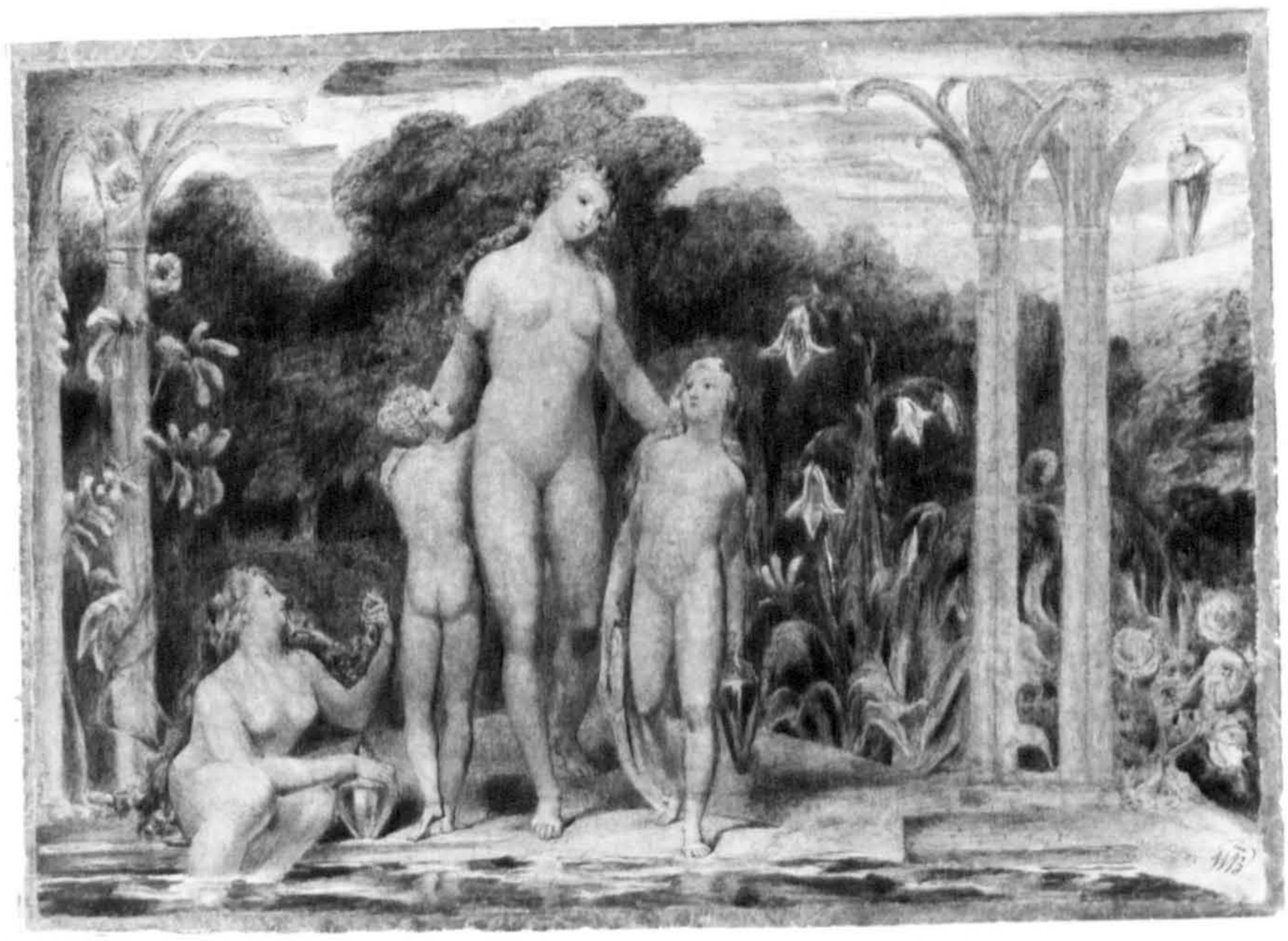


PLATE 3

PLATE 4

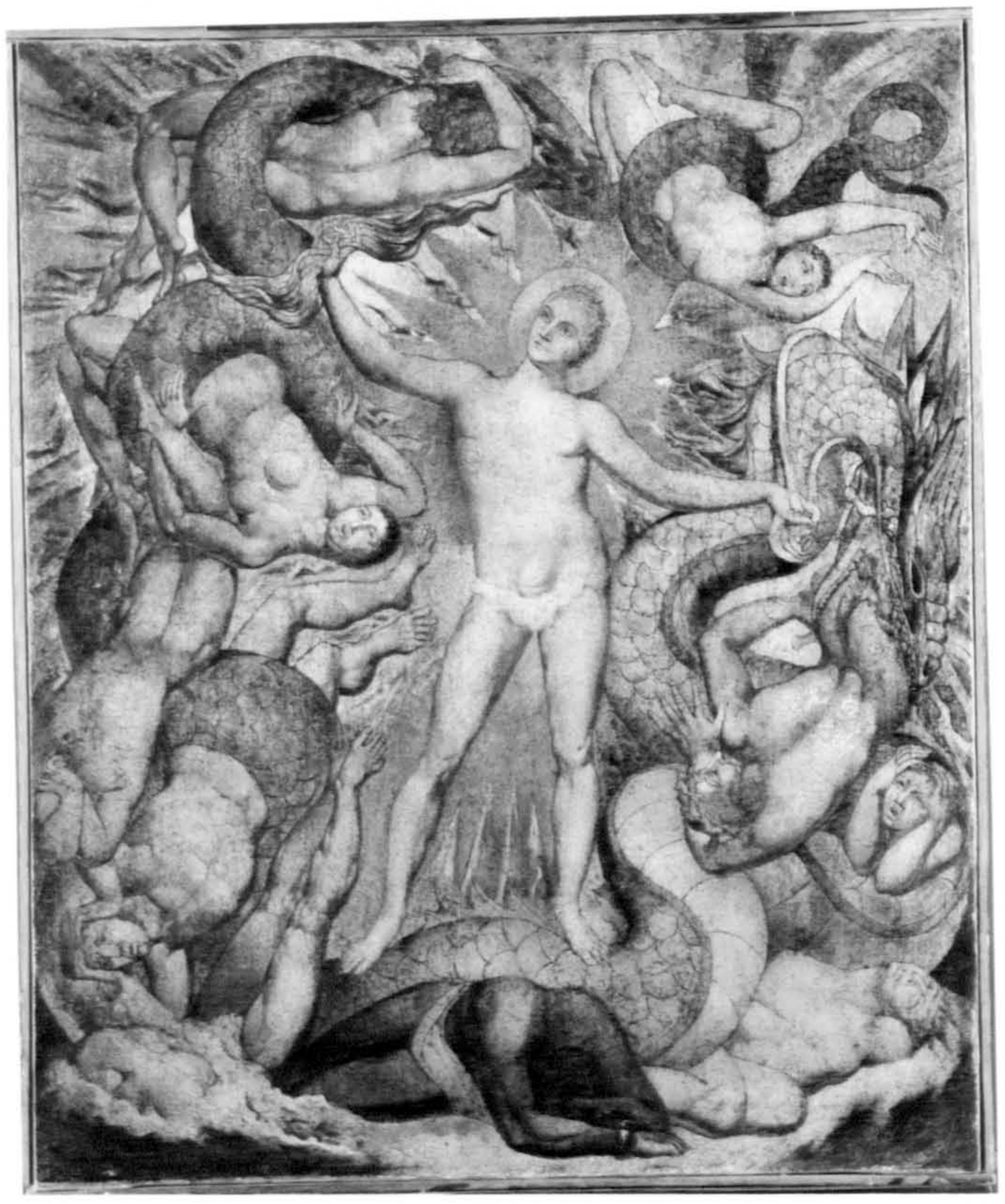


PLATE 5

PLATE 6

PLATE 7

PLATE 8

PLATE 9

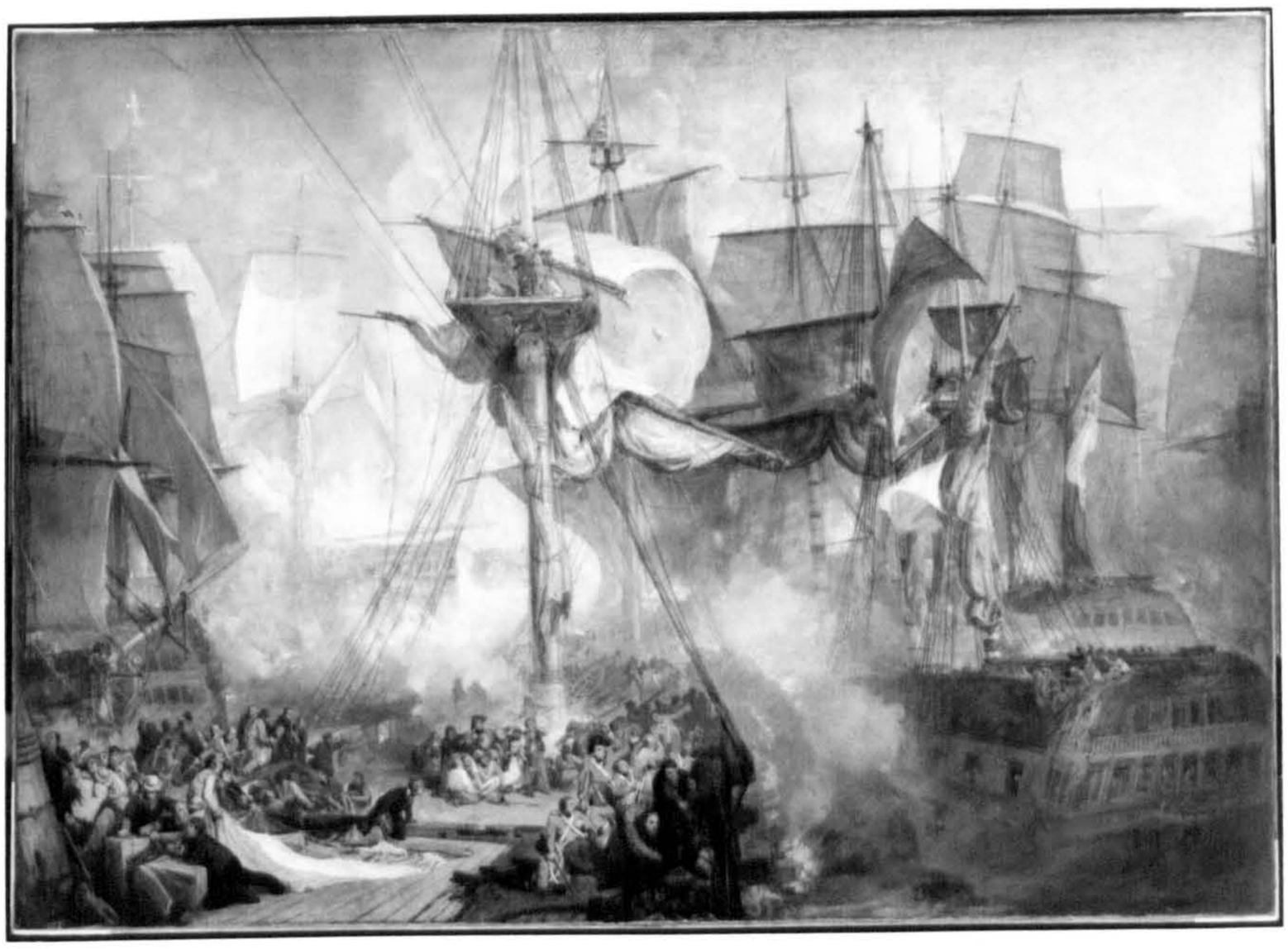


PLATE 10

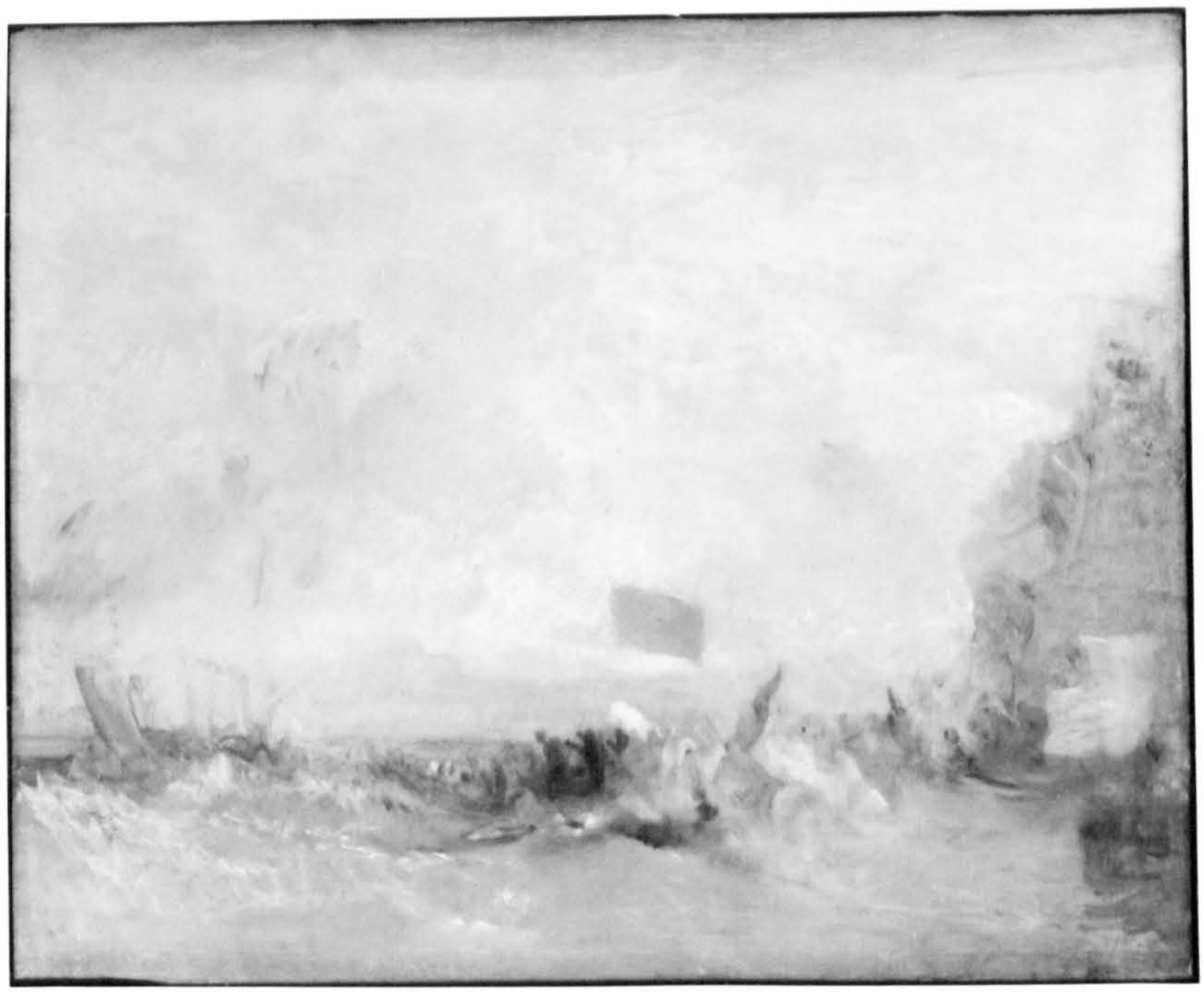


PLATE 11

PLATE 12

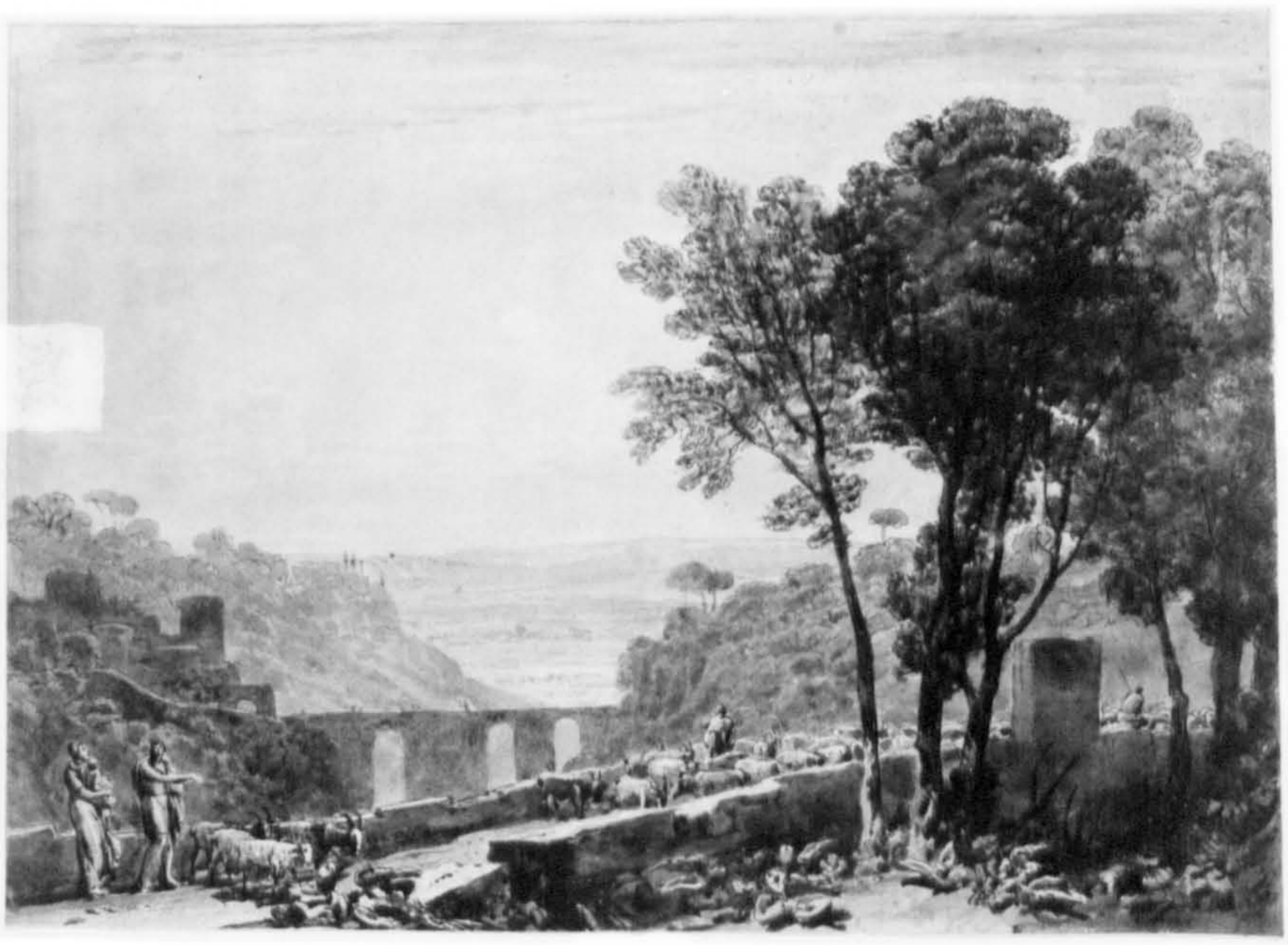


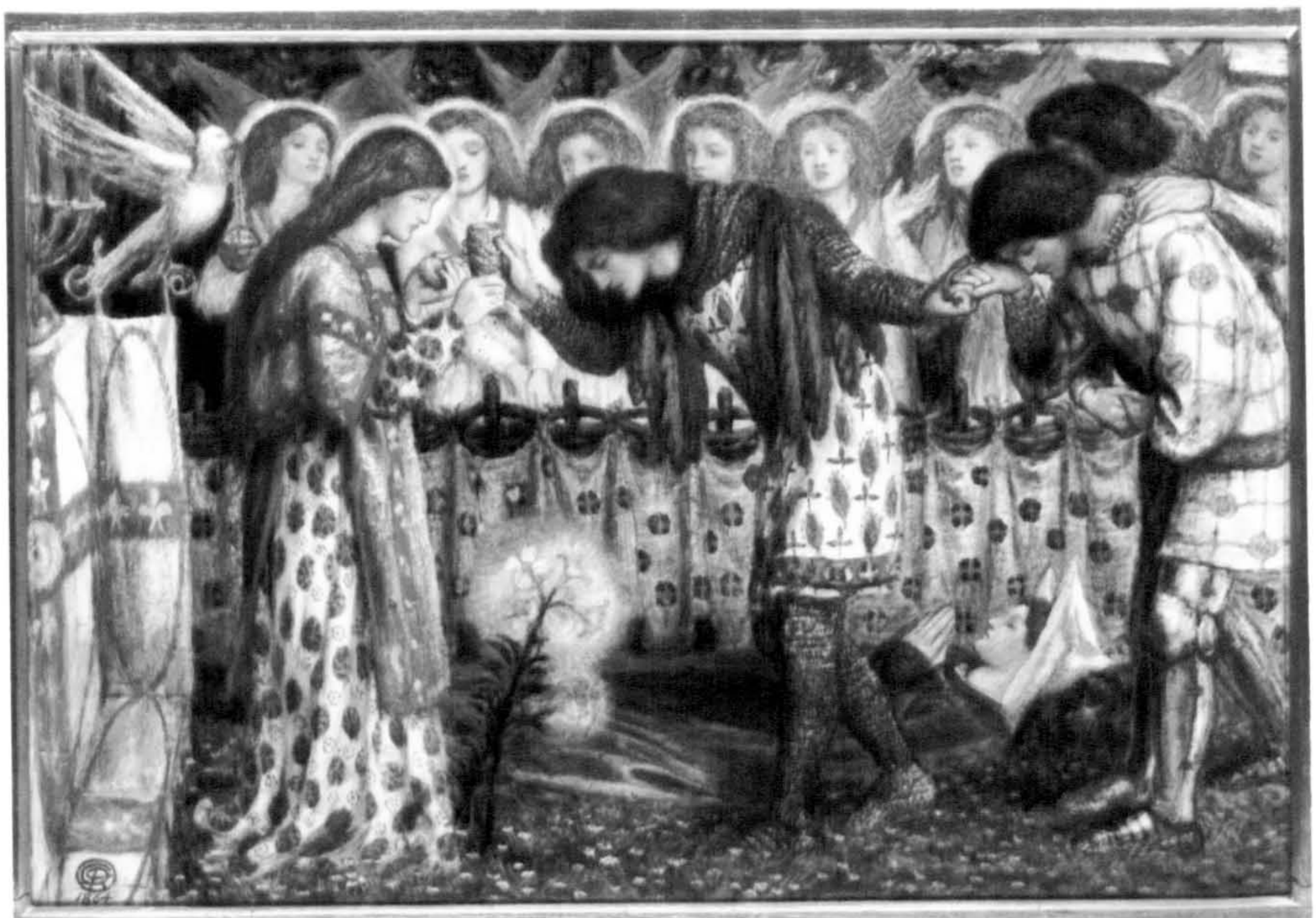
PLATE 13

PLATE 14

PLATE 15

PLATE 16

PLATE 17



How Sir Galahad, Sir Bors, & Sir Percival were fed with the Sanegreal; but Sir Percivals Sister died by the way.

APPENDIX 4

Chromatograms for Media Samples Removed from Works of Art

Figure A4.1: BLAKE - *Body of Christ Borne to the Tomb* (N01164). Protein and gum analyses of green paint, blue paint and priming samples.

Figure A4.2: BLAKE – *The Flight into Egypt* (L01778). Protein analysis of priming with pink paint and brown paint samples.

Figure A4.3: BLAKE – *Bathsheba at the Bath* (N03007). Gum analysis of priming and white/blue paint samples.

Figure A4.4: BLAKE – *The Ghost of a Flea* (N05889). Protein and gum analyses of priming, dark background paint and blue paint samples.

Figure A4.5: BLAKE – *The Agony in the Garden* (N05894). Protein and gum analyses of blue paint with priming samples.

Figure A4.6: BLAKE – *Spiritual Form of Nelson Guiding Leviathan* (N03006). Gum analyses of priming and brown paint samples.

Figure A4.7: BLAKE – *The Bard, from Gray* (N03551). Gum analysis of clear, glossy media samples.

Figure A4.8: TURNER – *George IV's Departure from the 'Royal George'* (N02880) and *The Bridge and Goats* (D08147). Protein and gum analyses of adhesive and paint medium samples.

Figure A4.9: ROSSETTI – *Dr. Johnson at the Mitre* (N03827) and *How Sir Galahad....* (N05234). Gum analysis of paint media samples.

Figure A4.10: KETEL – *Robert Smythe* (Ketel) and REYNOLDS – *Sir James Hodges* (N03545). Protein analysis of priming samples.

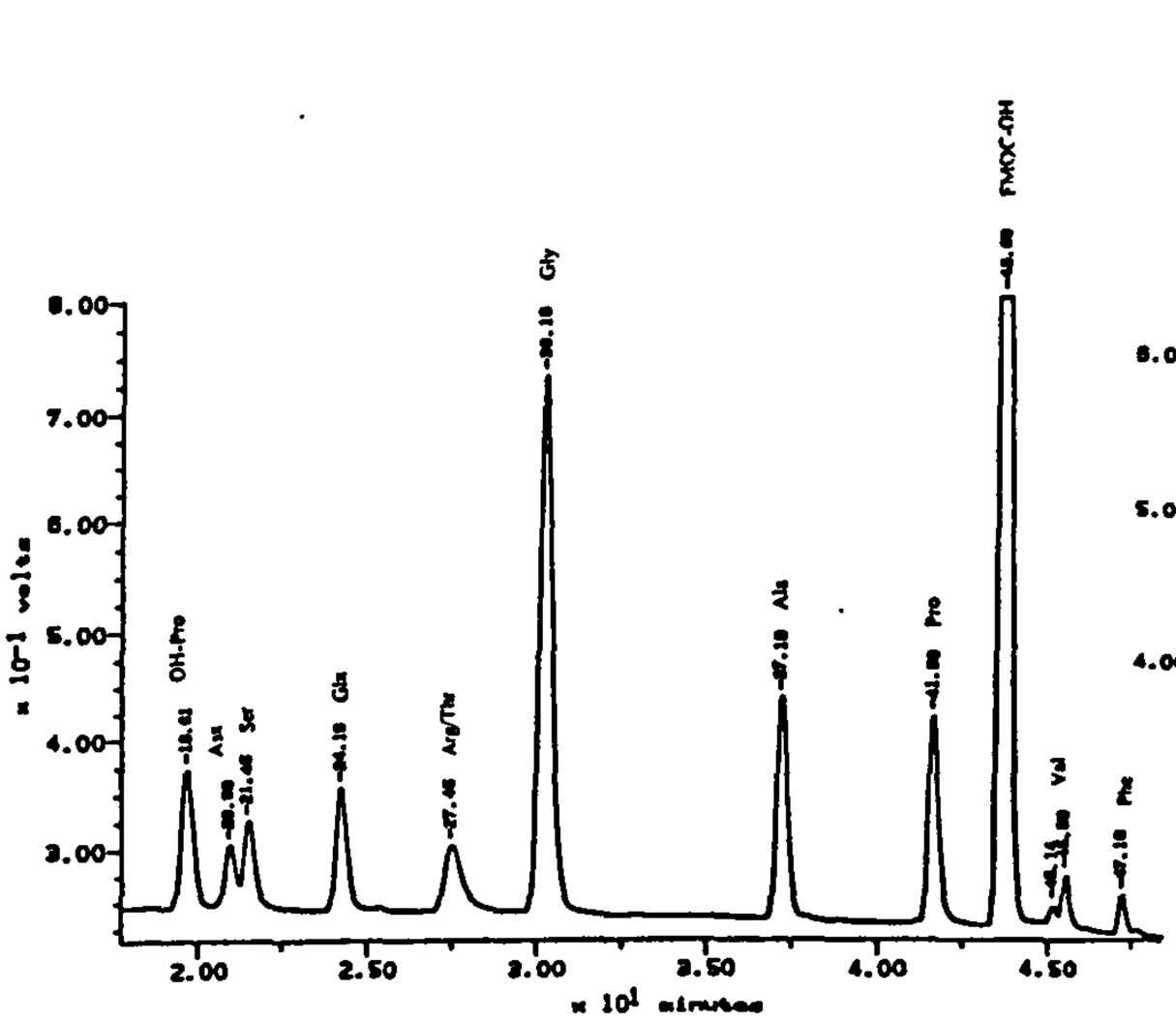
Figure A4.11: GRANT – *The Ass* (L01615). Protein analysis of priming sample.

Figure A4.12: Samples from Projects – MA1 and MA3. Protein analysis of lining adhesive and paint medium samples.

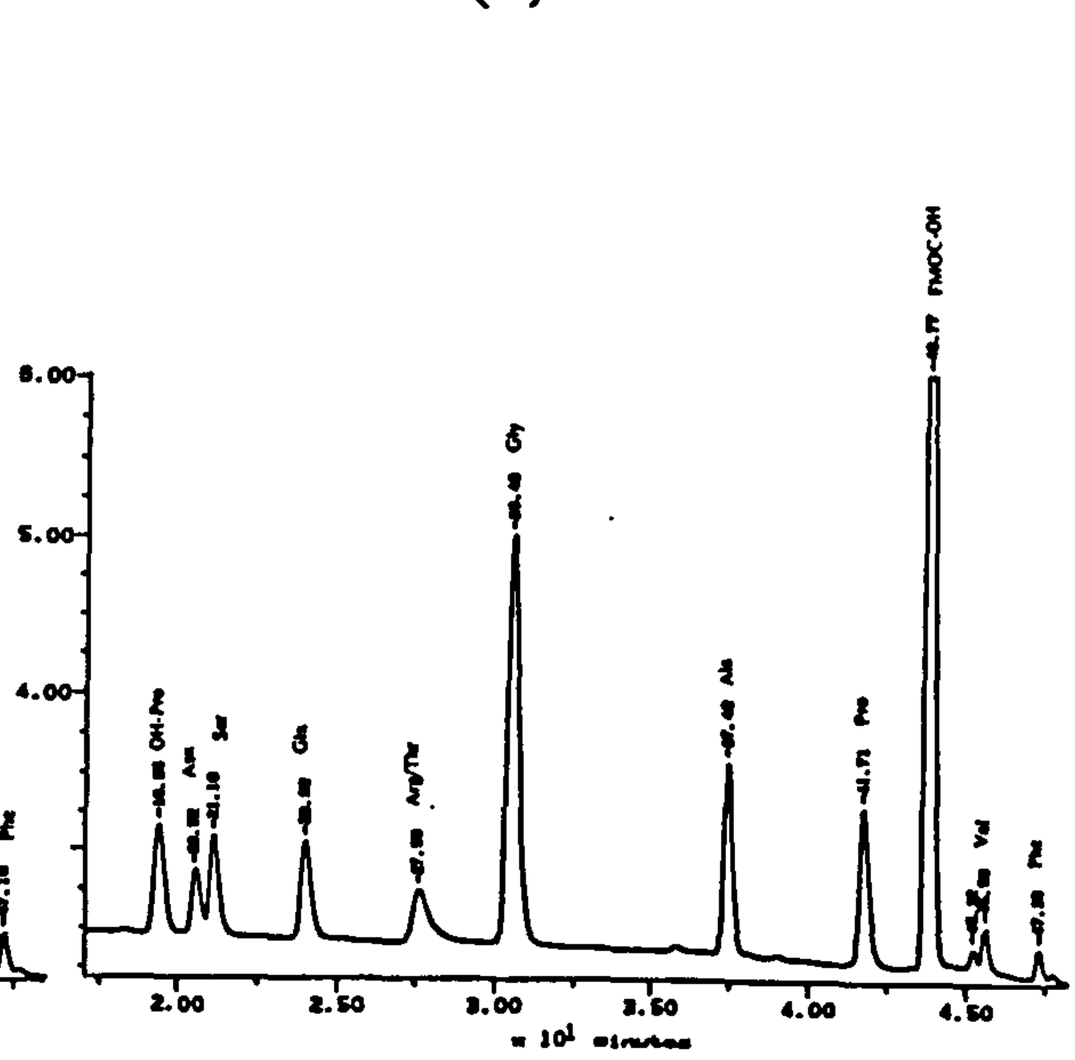
Figure A4.1: BLAKE - *Body of Christ Borne to the Tomb* (N01164)

(A) HPLC Green Paint; (B) HPLC Blue Paint; (C) GC Green Paint; (D) GC Priming

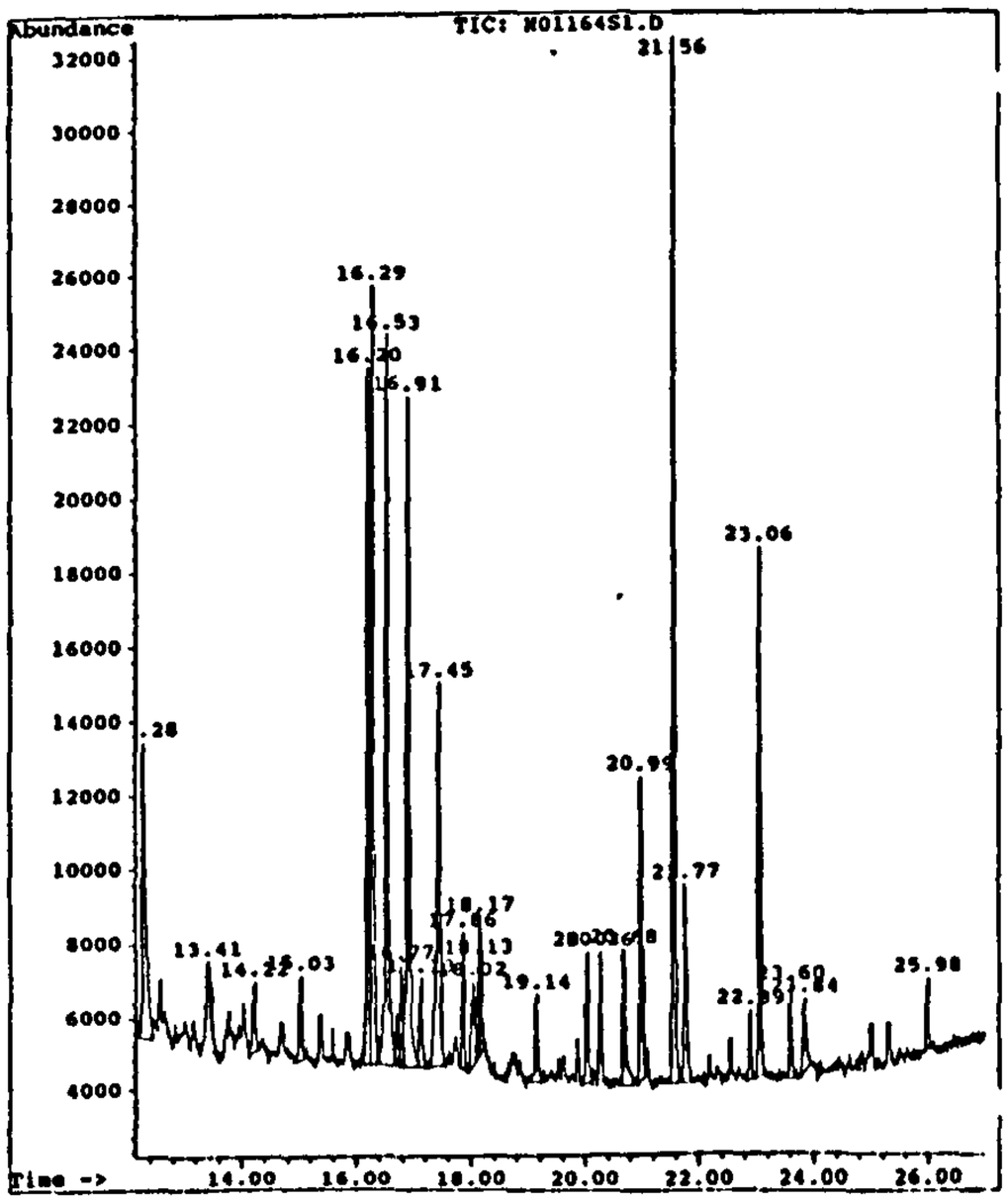
(A)



(B)



(C)



(D)

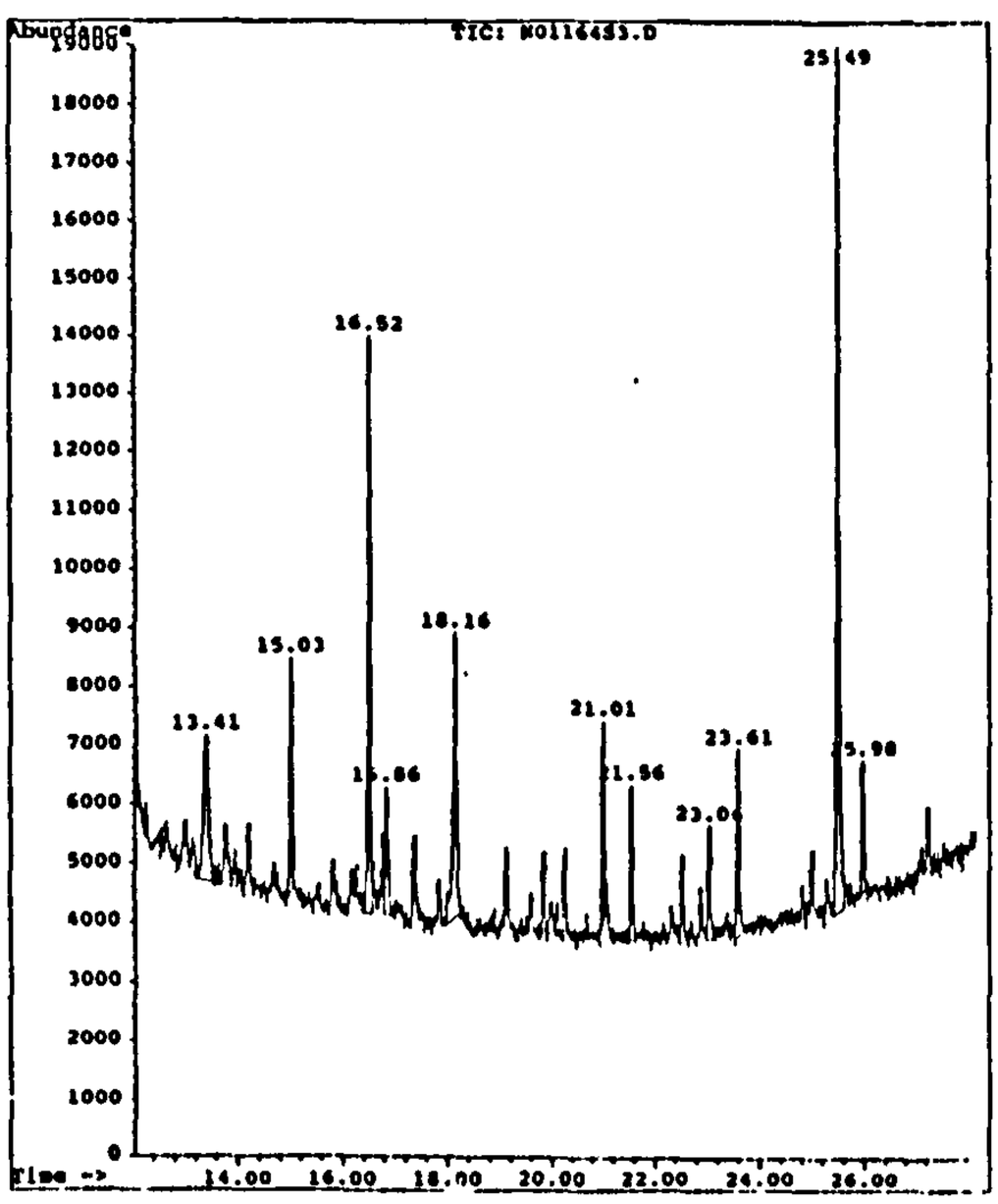


Figure A4.2: BLAKE – *The Flight into Egypt* (L01778)

(A) HPLC Priming & Pink Paint; (B) HPLC Brown Paint

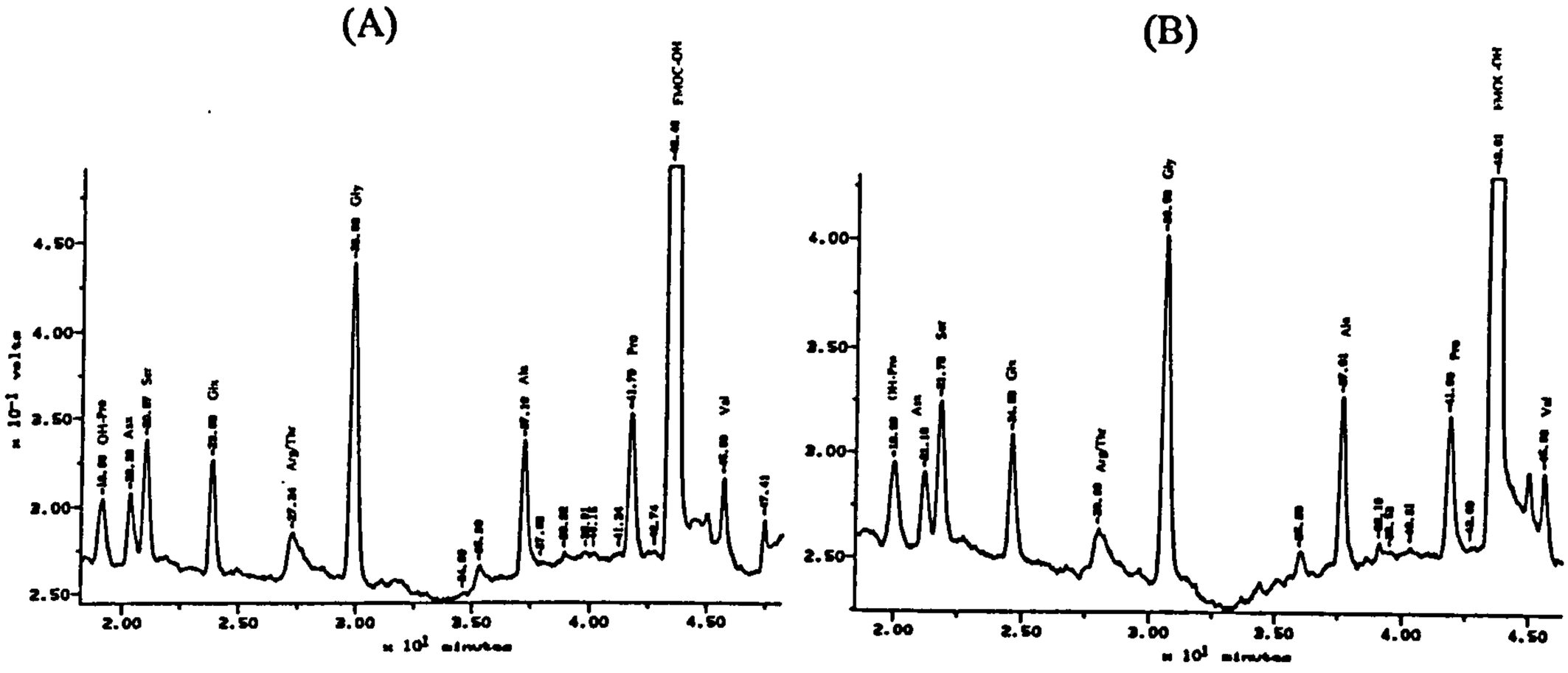


Figure A4.3: BLAKE – *Bathsheba at the Bath* (N03007)

(A) GC Priming; (B) GC White/Blue Paint

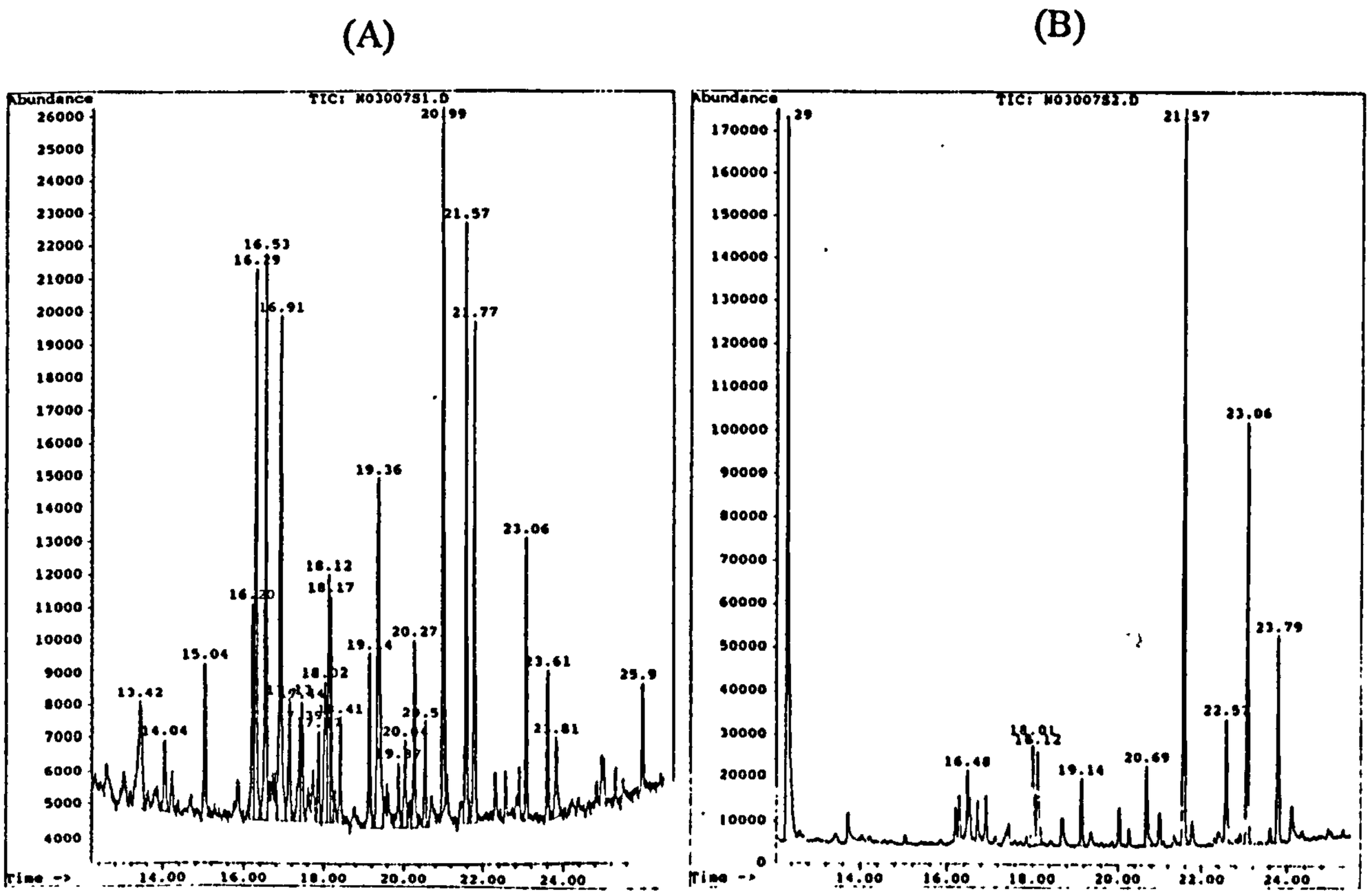
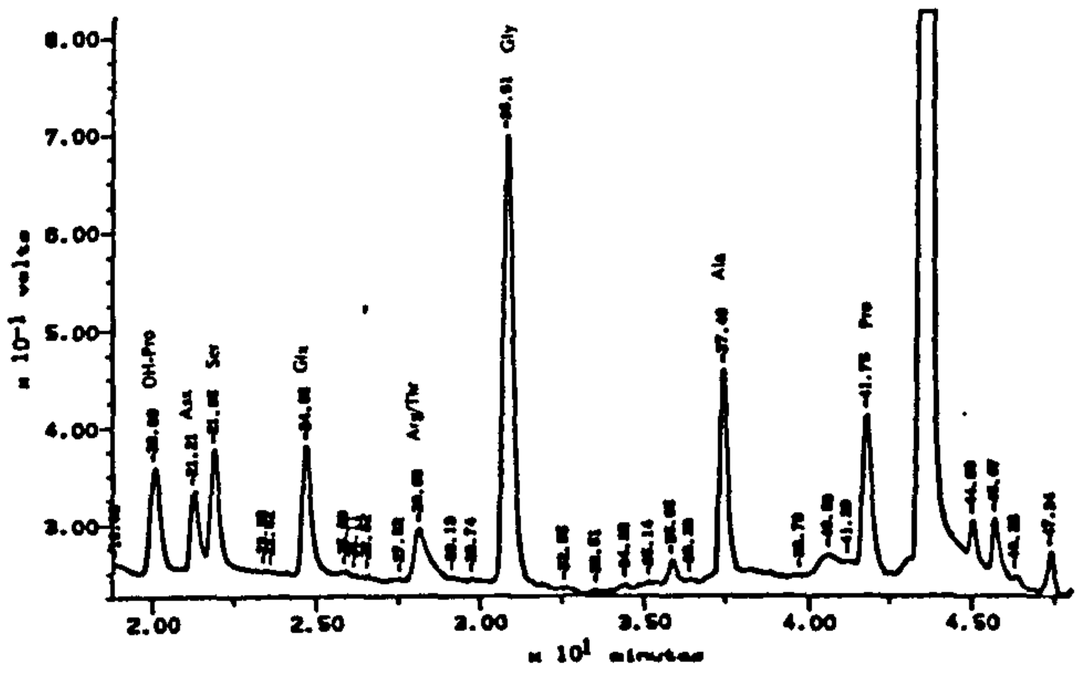


Figure A4.4: BLAKE – *The Ghost of a Flea* (N05889)

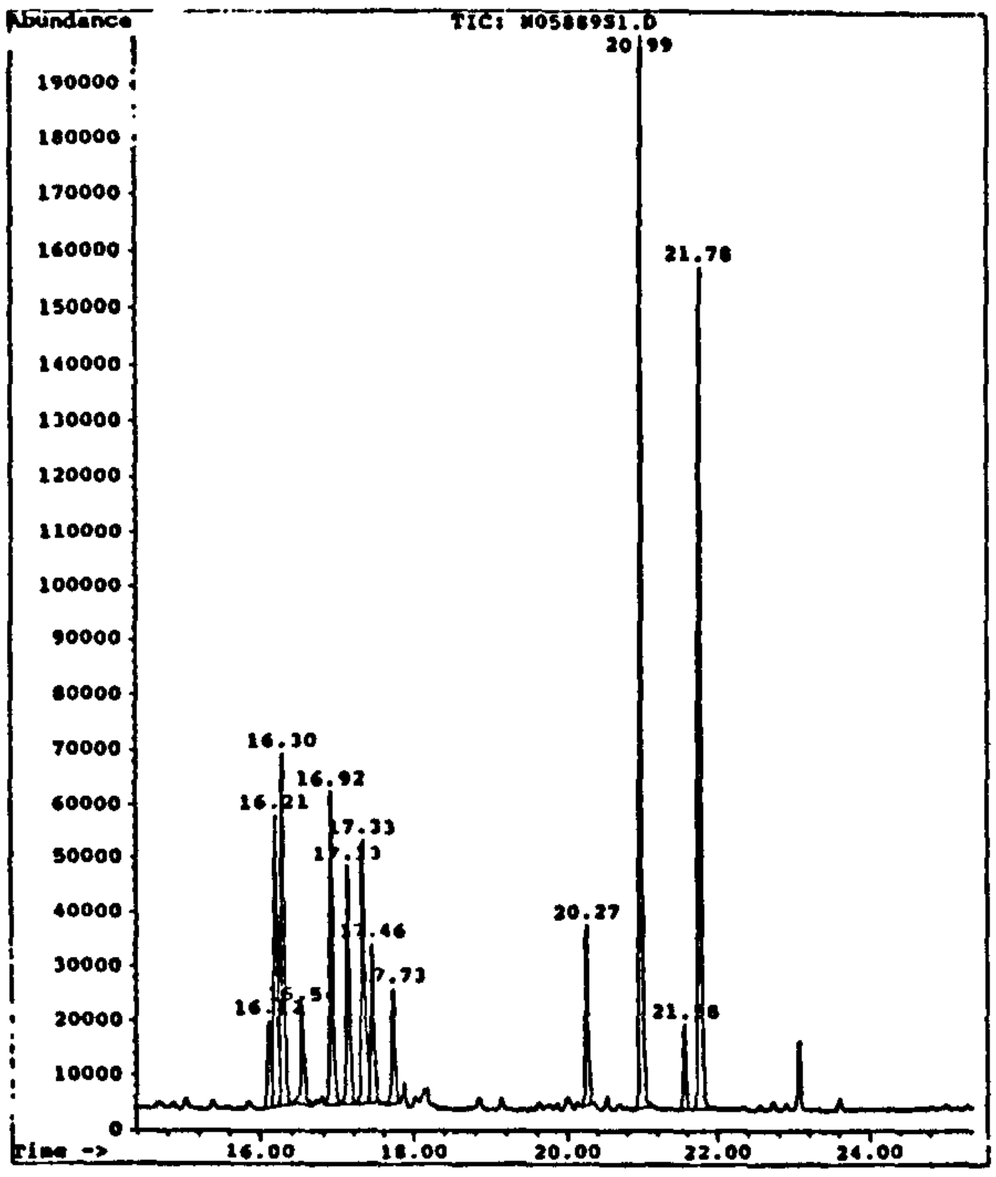
(A) HPLC Priming; (B) GC Priming; (C) GC Dark Background Paint;

(D) GC Blue Paint

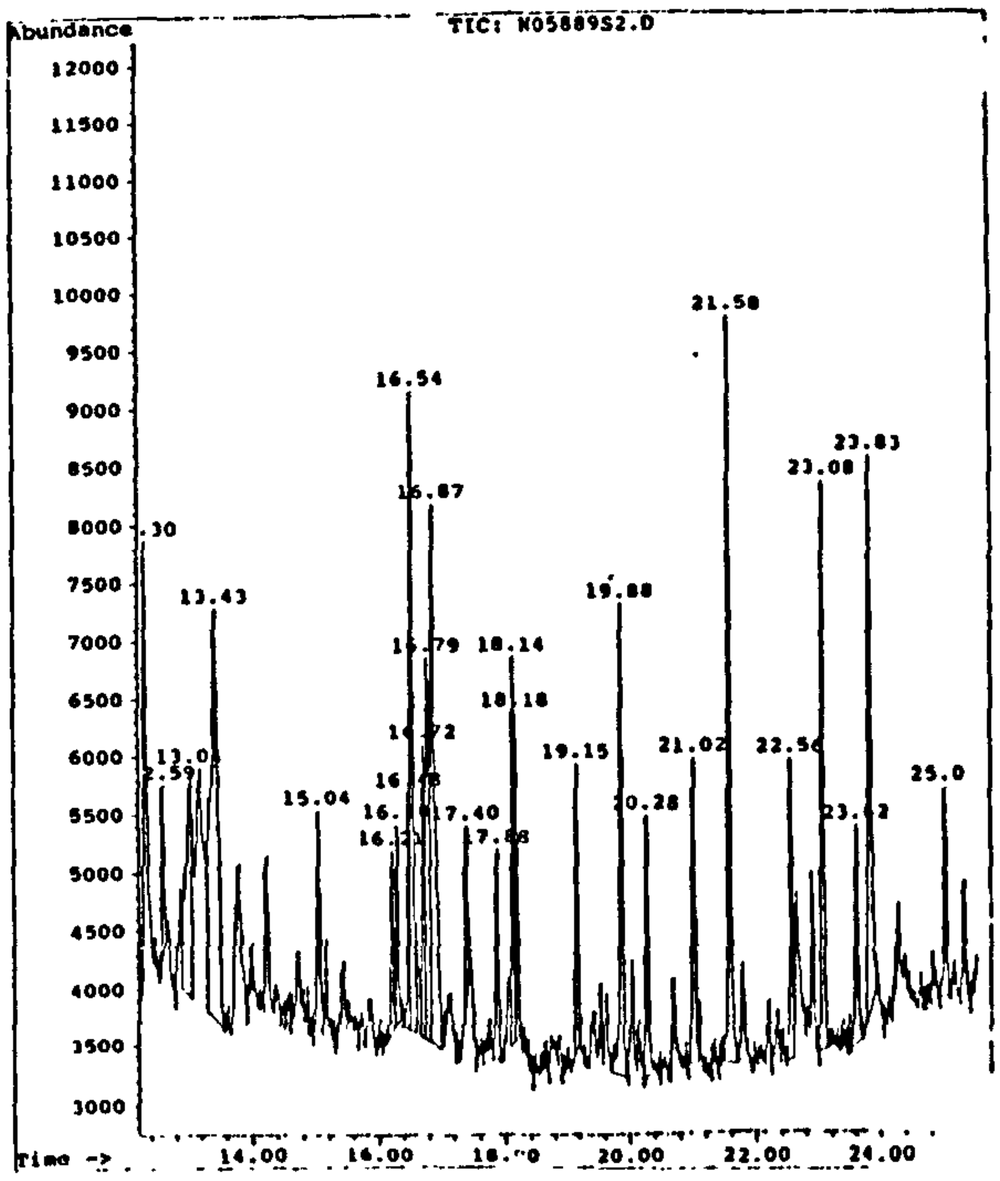
(A)



(B)



(C)



(D)

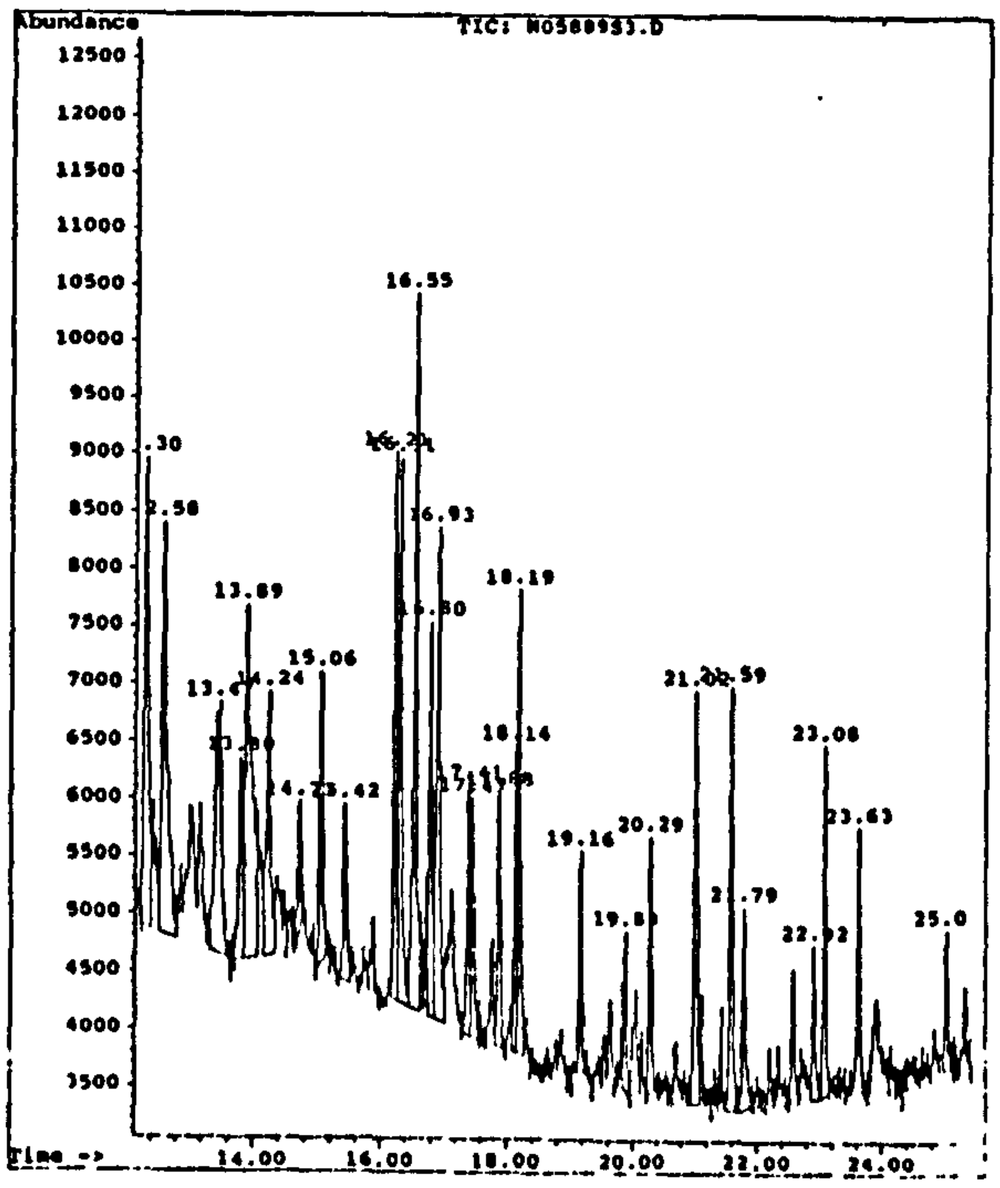
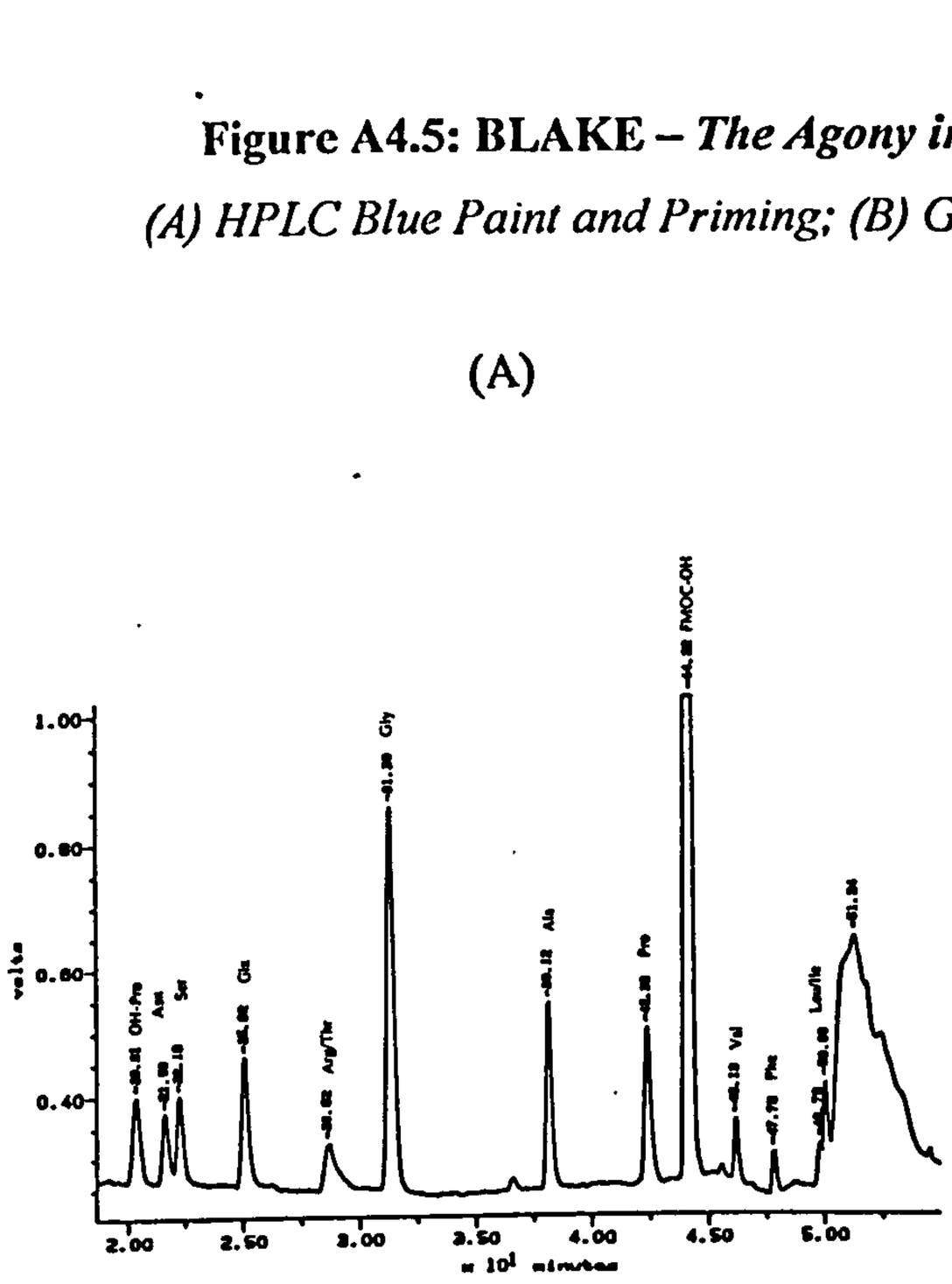


Figure A4.5: BLAKE – *The Agony in the Garden* (N05894)

(A) HPLC Blue Paint and Priming; (B) GC Blue Paint and Priming

(A)



(B)

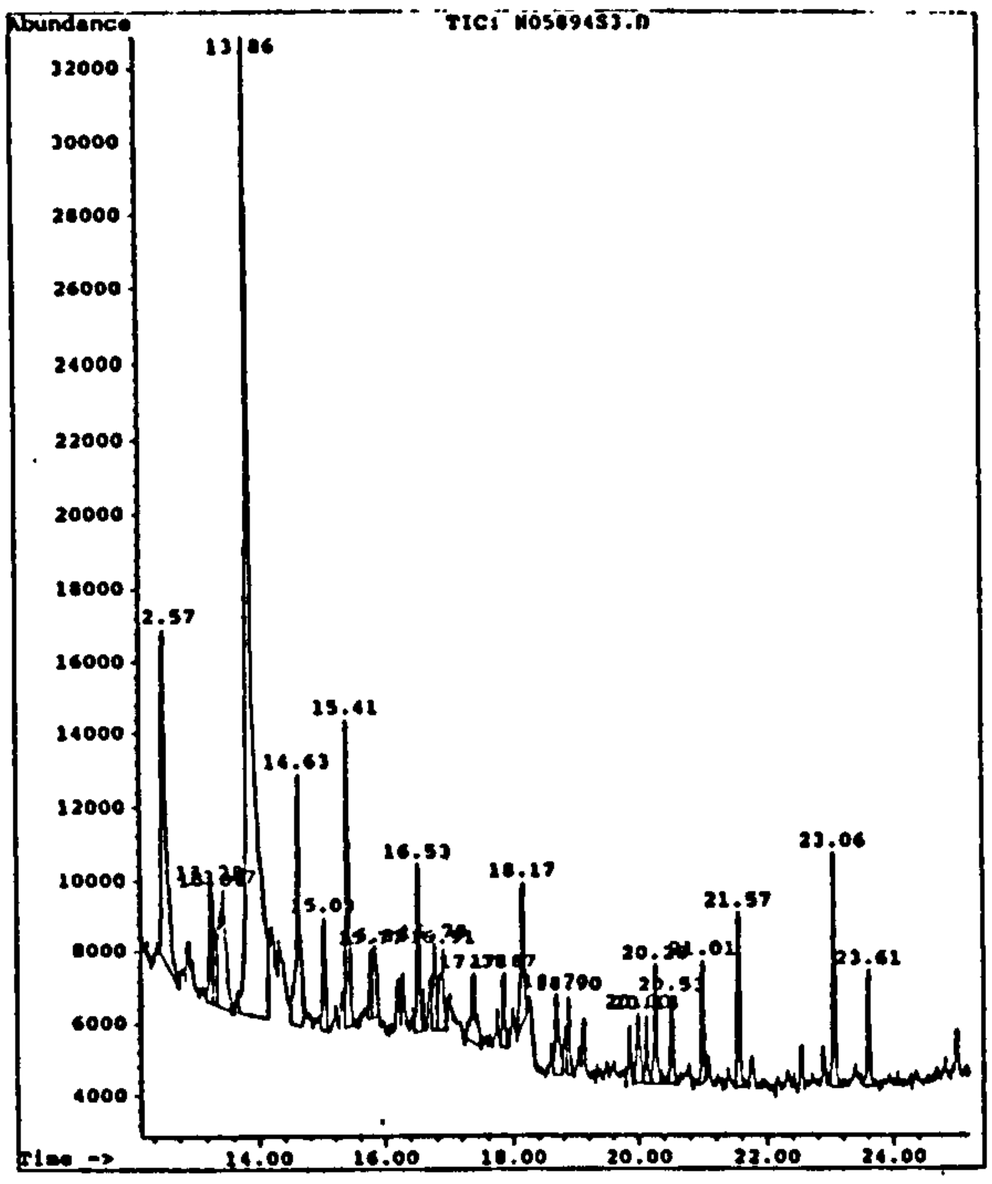
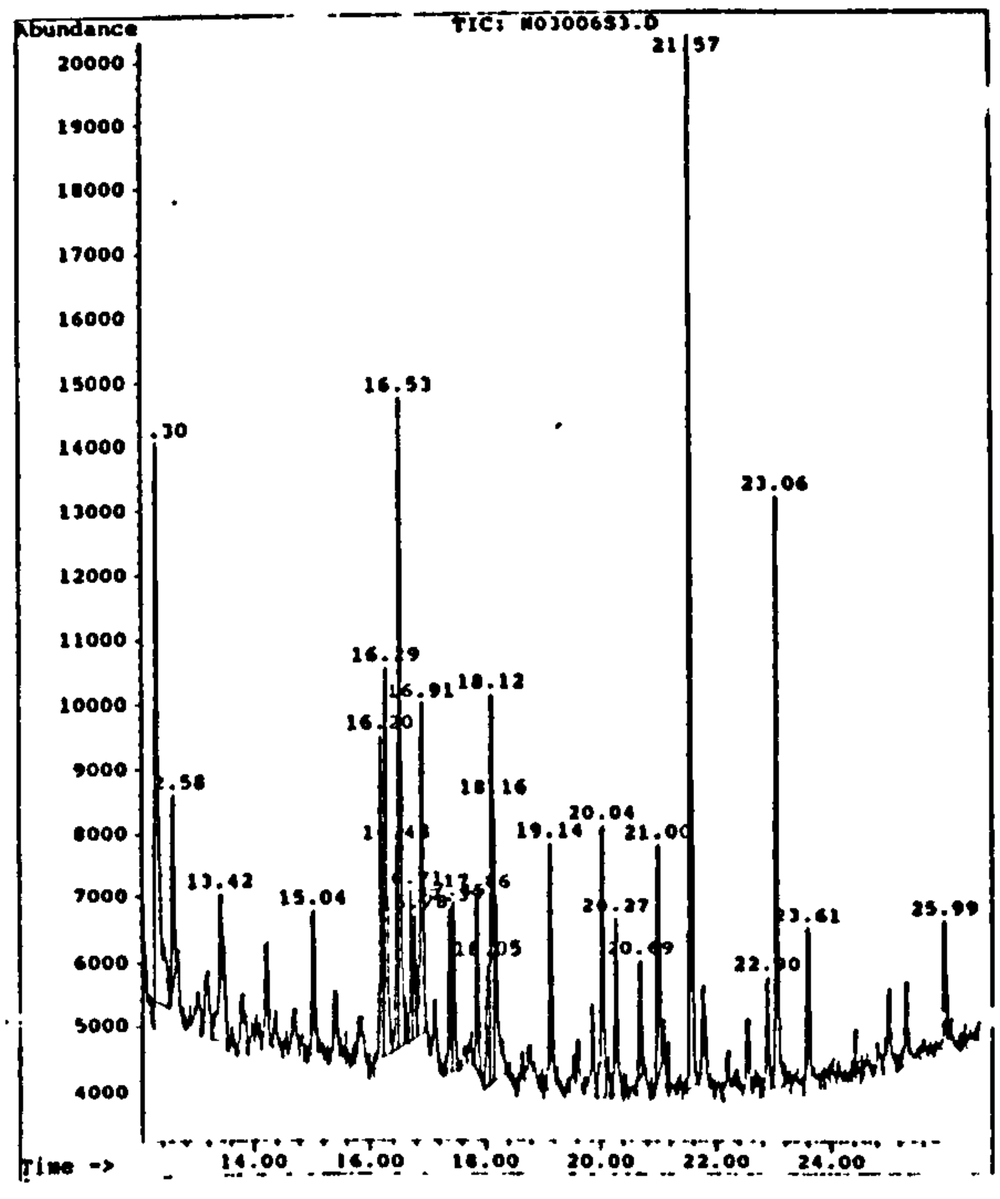


Figure A4.6: BLAKE – *Spiritual Form of Nelson Guiding Leviathan* (N03006)

(A) GC White Priming; (B) GC Brown Paint

(A)



(B)

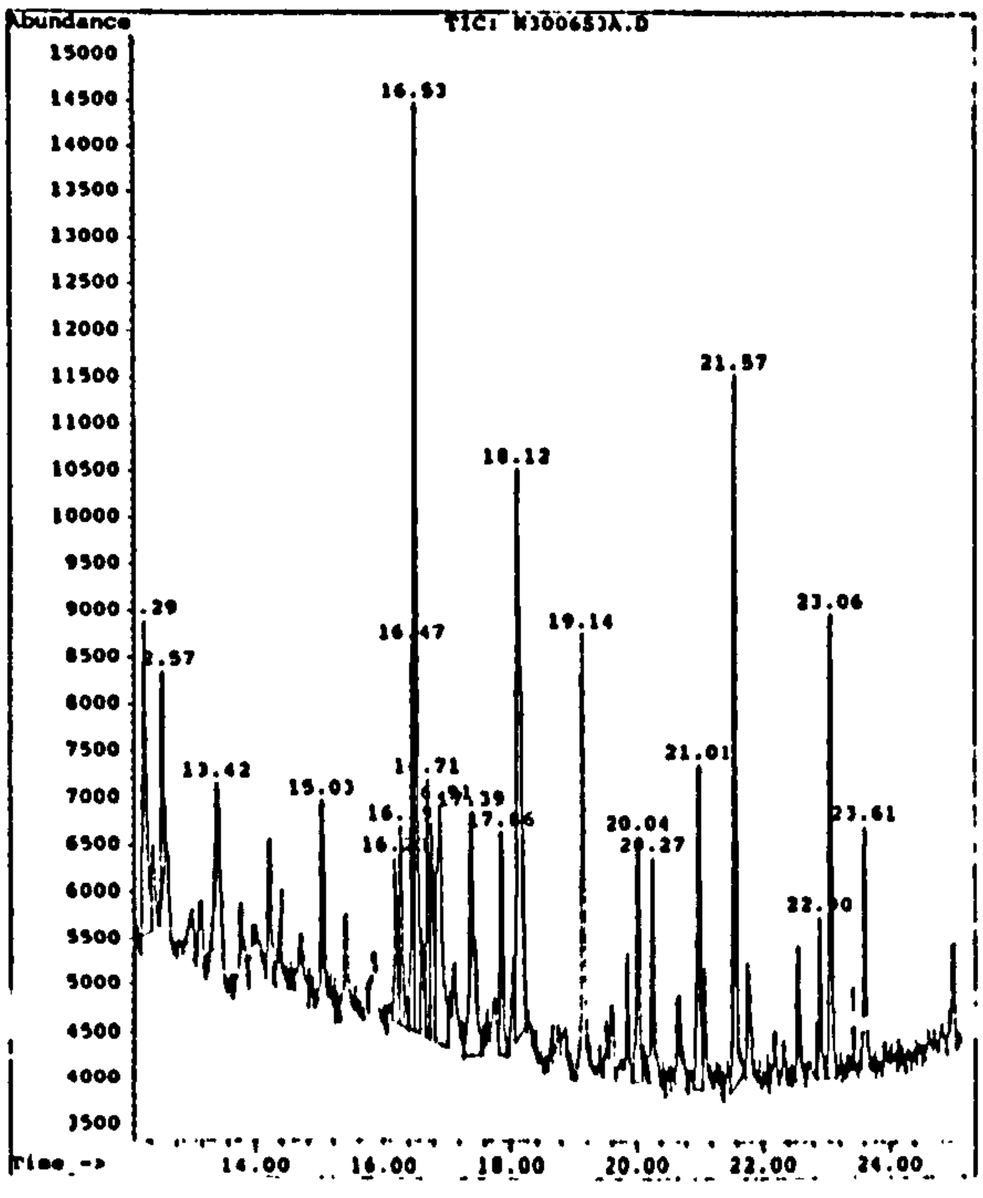
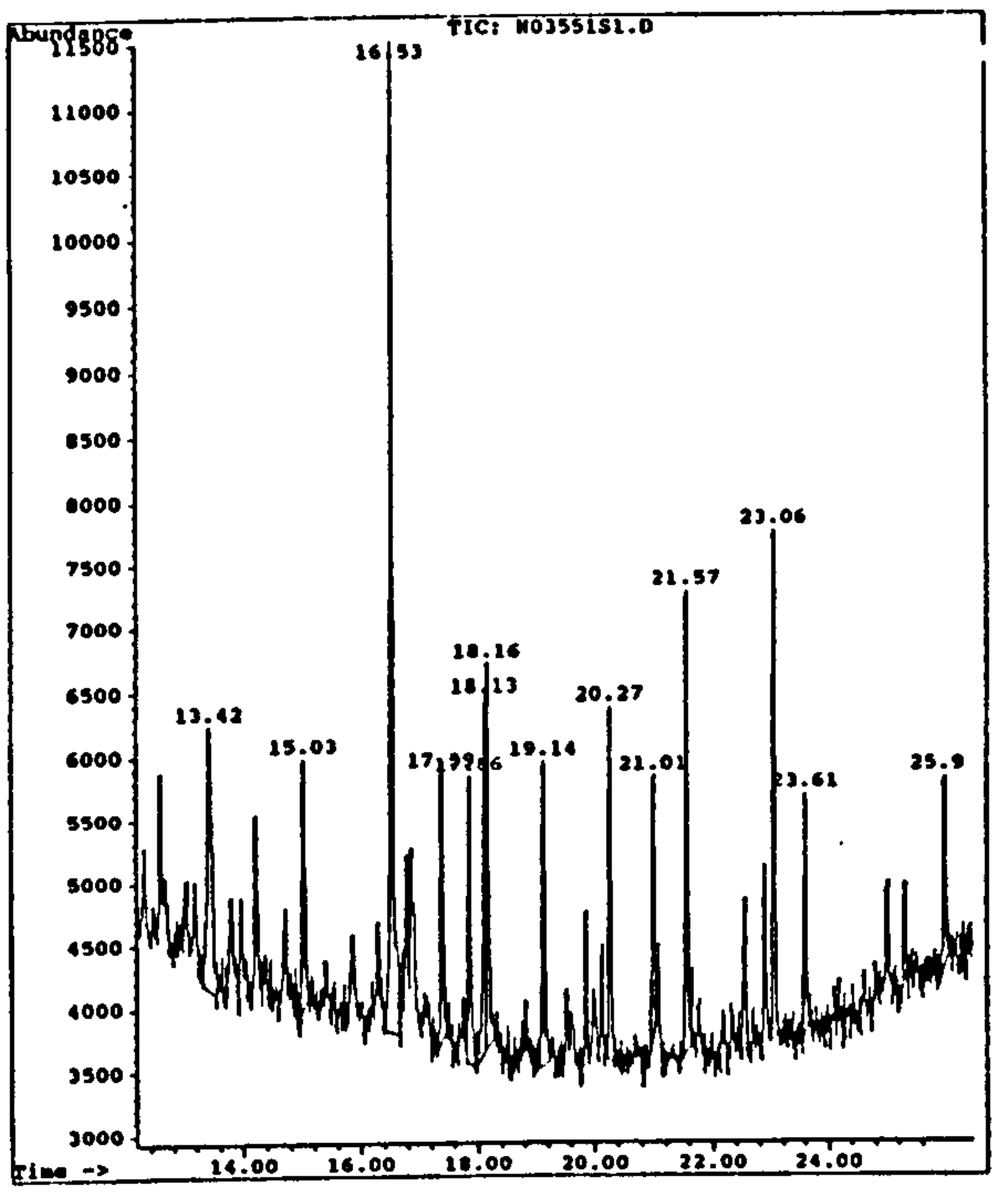


Figure A4.7: BLAKE – *The Bard, from Gray* (N03551)

(A) GC Clear Glossy Medium (S1); (B) GC Clear Glossy Medium (S2)

(A)



(B)

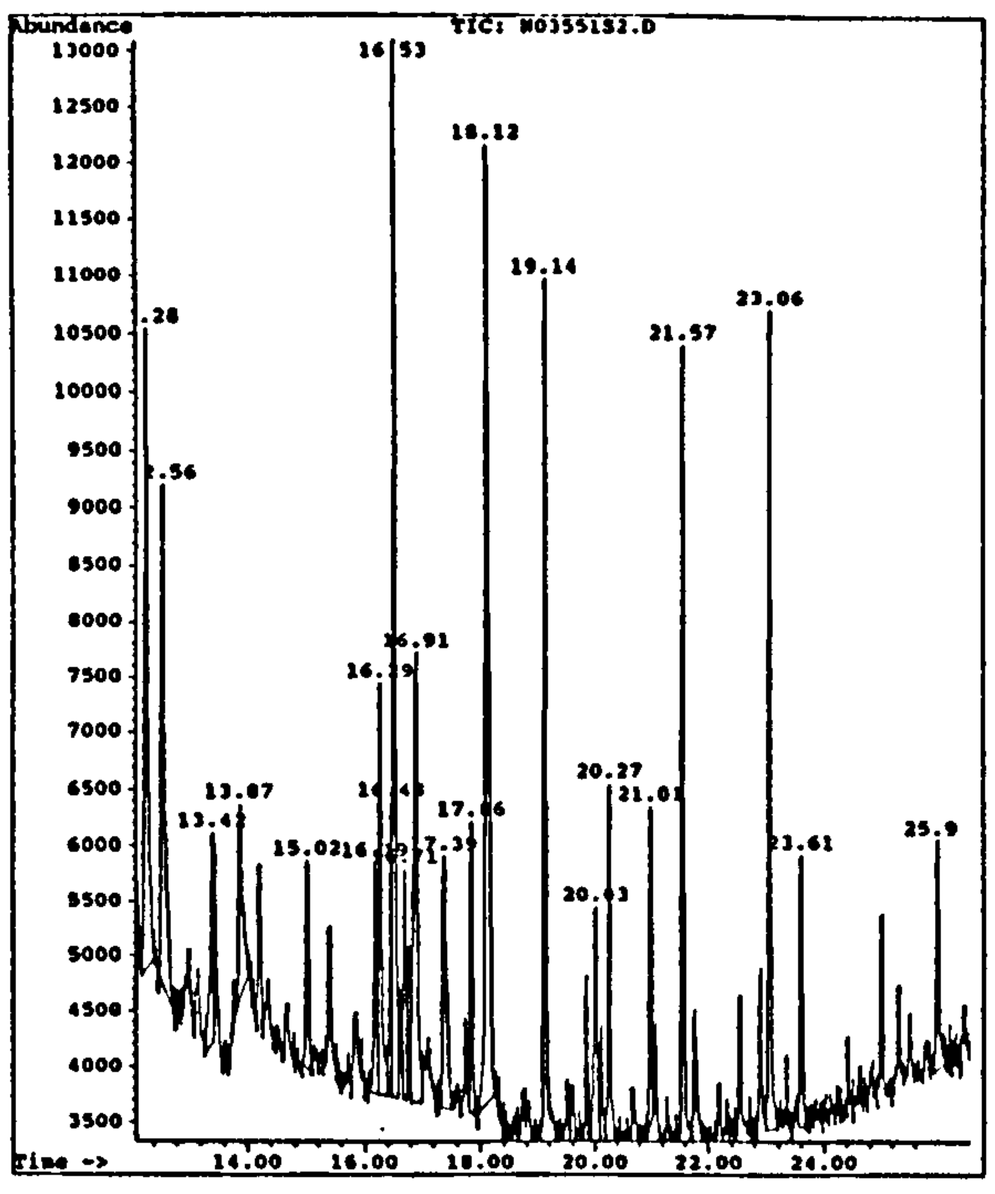
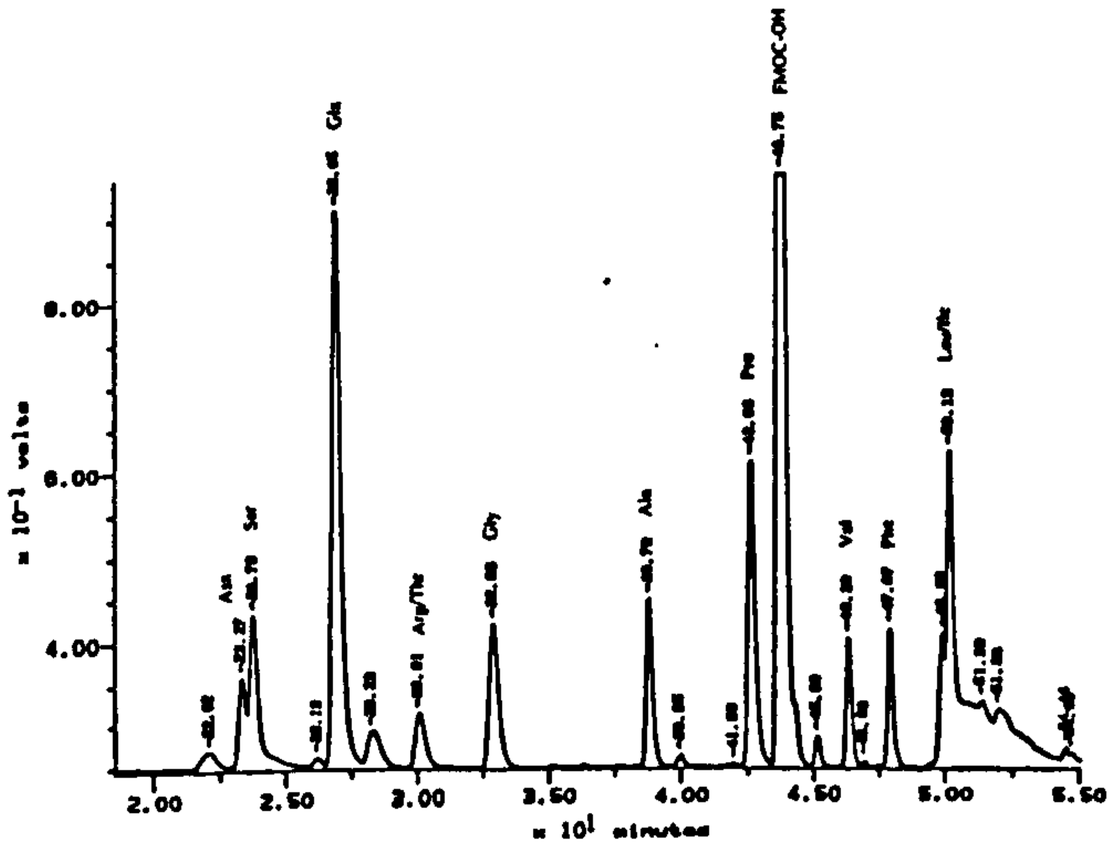


Figure A4.8: TURNER – *George IV's Departure from the 'Royal George'* (N02880) and *The Bridge and Goats* (D08147)

(A) HPLC Adhesive from N02880; (B) GC Paint from D08147

(A)



(B)

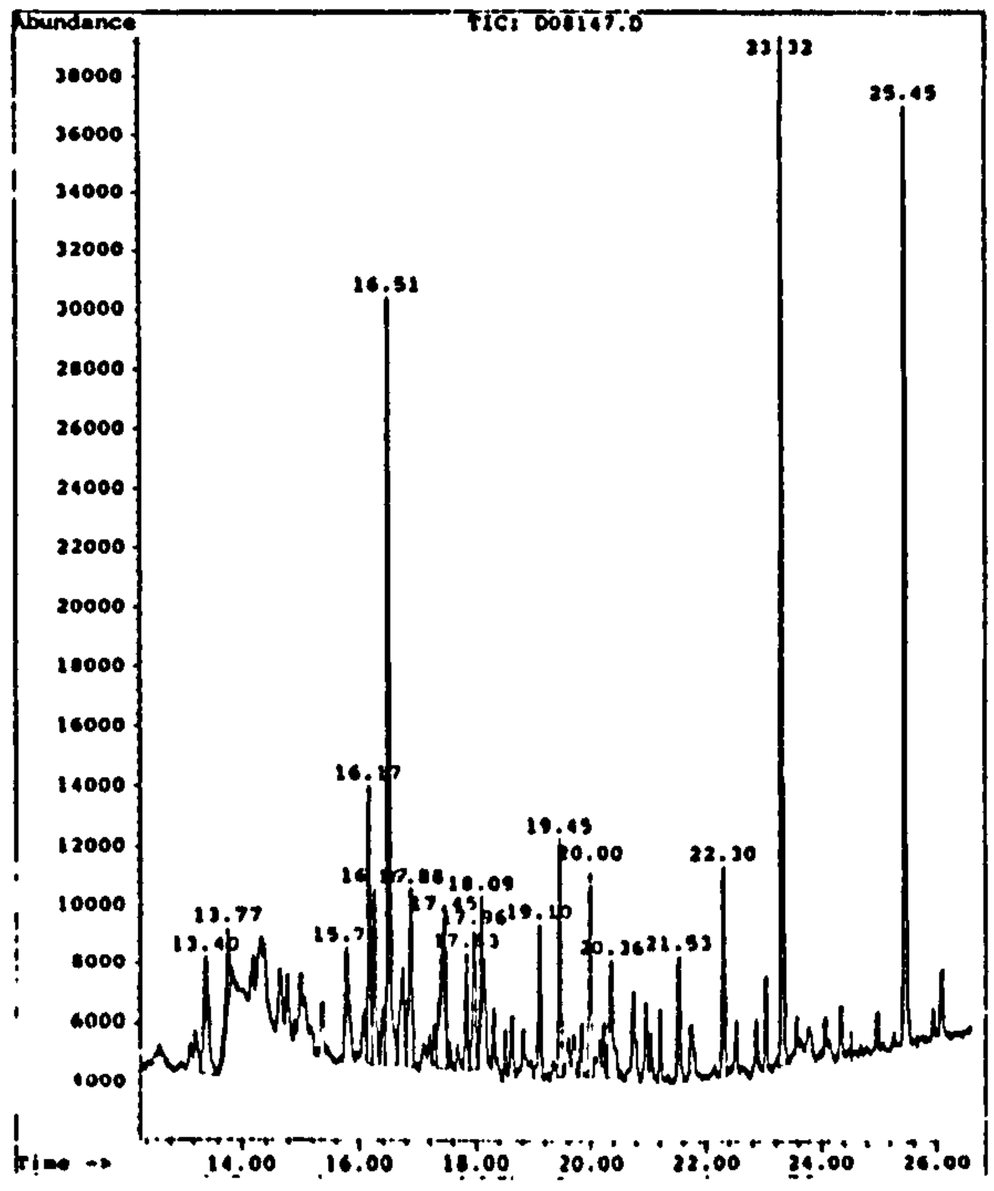


Figure A4.9: ROSSETTI – *Dr. Johnson at the Mitre* (N03827) and *How Sir Galahad....* (N05234)

(A) GC Paint from N03827; (B) GC Red Paint from N05234

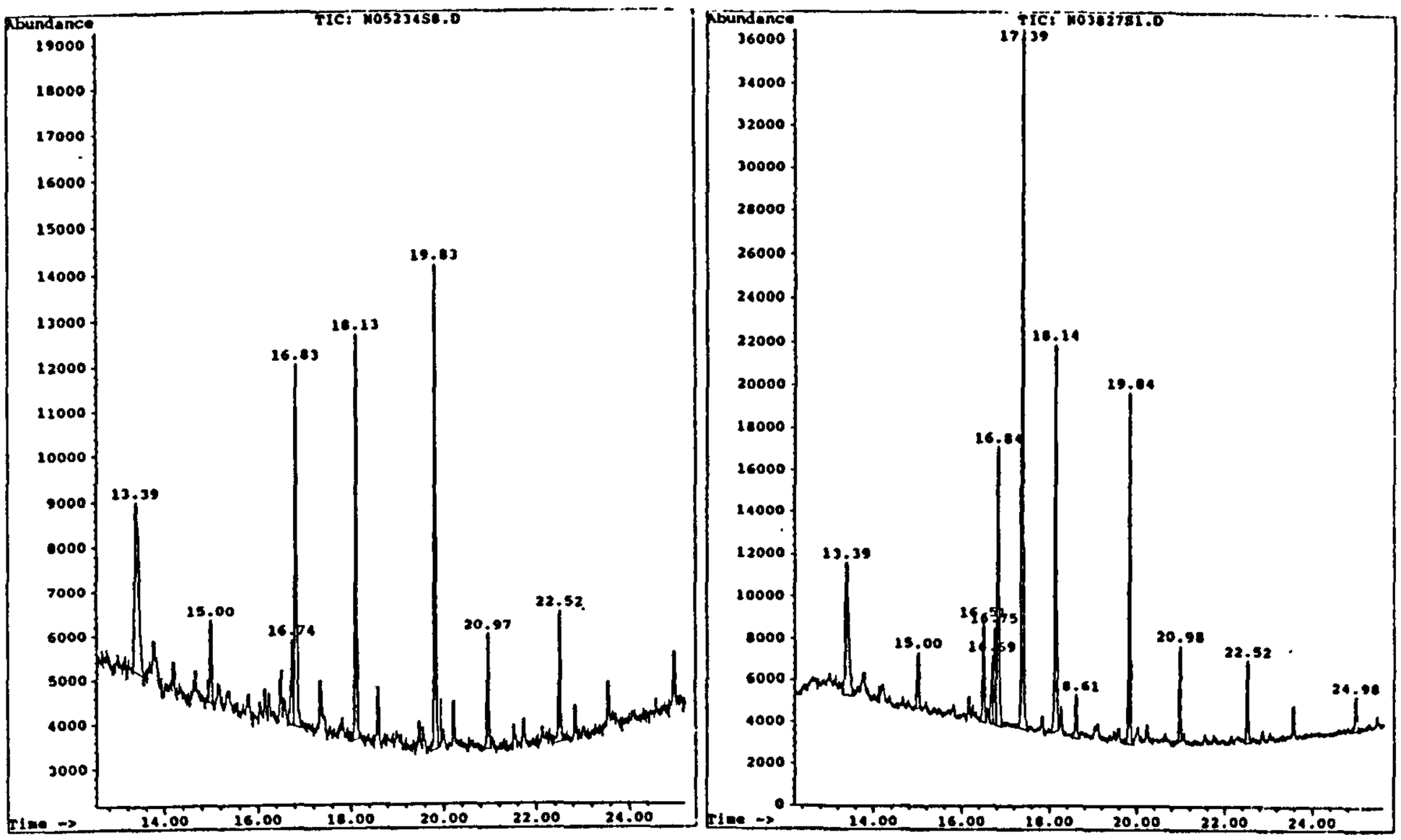


Figure A4.10: KETEL – *Robert Smythe* (Ketel) and REYNOLDS – *Sir James Hodges* (N03545)

(A) HPLC Priming from Ketel; (B) HPLC Priming from N03545

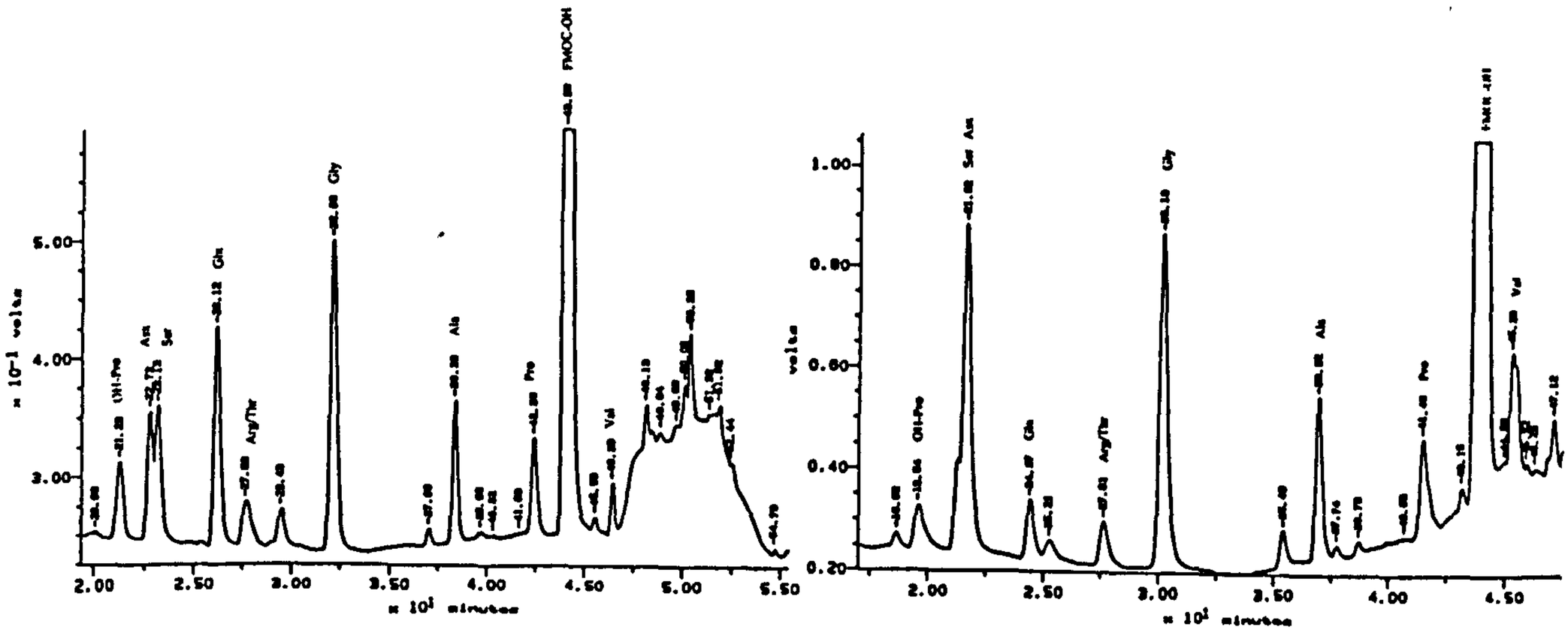


Figure A4.11: GRANT – *The Ass* (L01615)

HPLC Priming

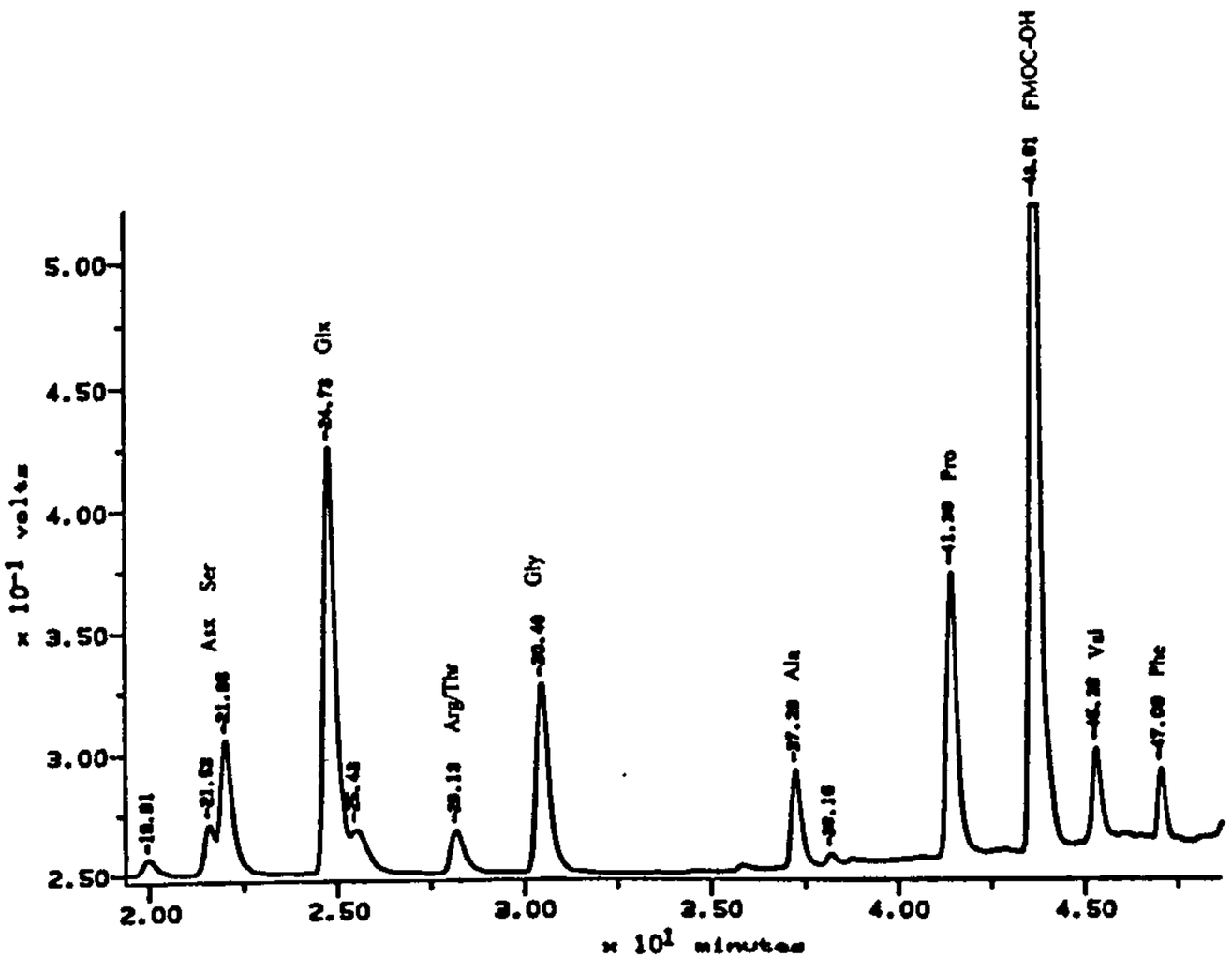
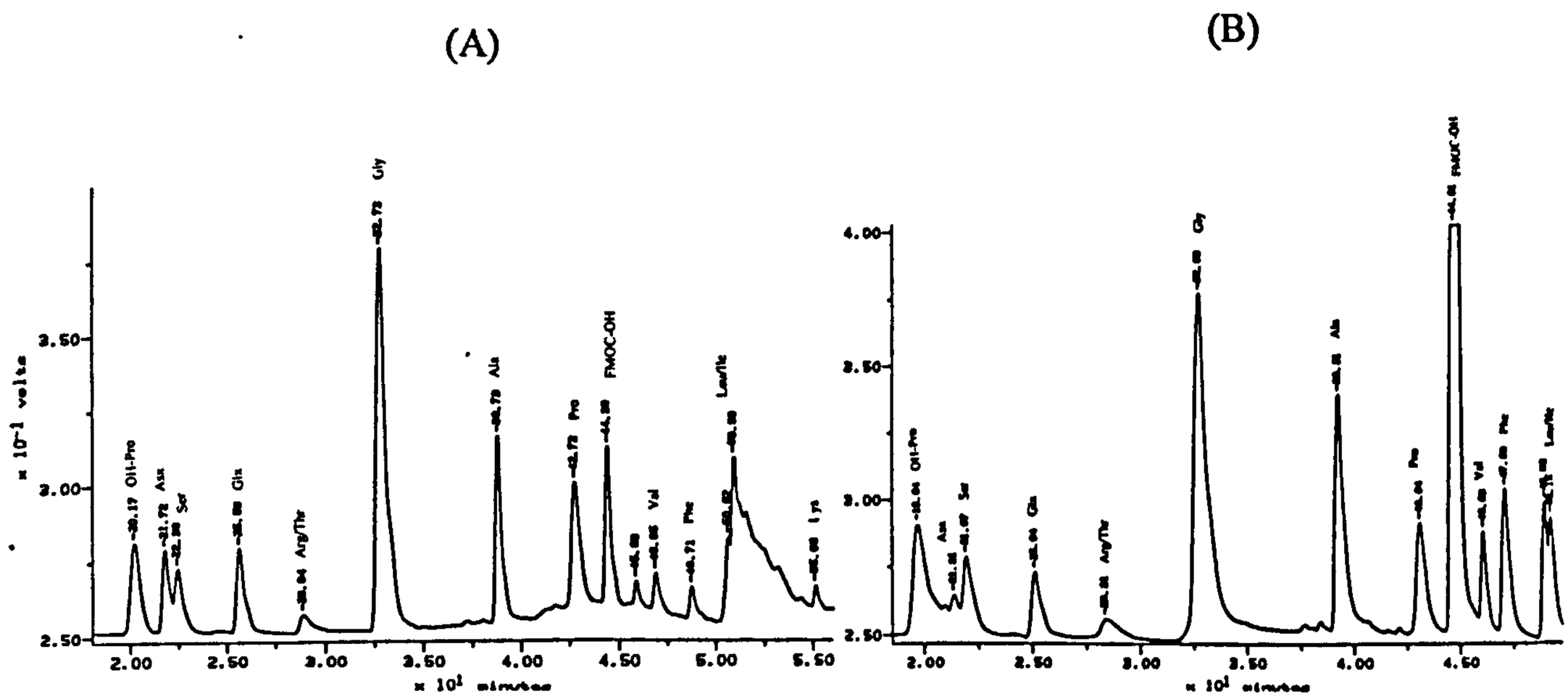


Figure A4.12: Samples from Projects – MA1 and MA3

(A) HPLC Lining Adhesive; (B) HPLC Media Sample



APPENDIX 5

Chemometrics

The following tables contain experimental data processed using *Statistica*, the computer software package for pattern recognition, and numerical results obtained from the analysis of that data.

Table A1: *Training Set Data and Group Classifications for Standard Proteinaceous Media.*

Table A2: *Regression Weights and Constants for Variables in Each Group.*

Table A3: *Means of Canonical Roots for Training Set.*

Table A4: *Data for Discriminant Analysis of Test Samples of Pure and Mixed Proteinaceous Media.*

Table A5: *Classification Scores for Test Samples.*

Table A6: *Experimental Data for Suspected Mixed Proteinaceous Media Samples.*

Table A7: *Classification Scores for Suspected Mixed Proteinaceous Media Samples.*

Table A8: *Discriminant Analysis – Statistical Summary.*

Table A9: *Training Set Data and Group Classification for Standard Gum Media.*

Table A10: *Regression Weights and Constants for Variables in Each Group.*

Case Name	OH-Pro	Ser	Asx	Glx	Arg/Thr	Gly	Ala	Pro	Val	Phe	Leu/Ile	Lys	Group
1	0.001	9.26	12.08	14.99	5.88	6.95	8.07	5.76	6.48	4.25	13.09	11.69	Yolk
2	0.0011	9.51	12.47	13.04	5.9	6.59	8.87	5.95	6.57	3.73	14.07	12.56	Yolk
3	0.0012	10.22	13.24	14.14	6.24	7.26	9.08	5.42	6.29	3.57	14.34	8.97	Yolk
4	0.0013	12.39	14.64	14.45	6.45	9.06	9.81	5.67	4.02	0.005	16.3	7.19	Yolk
5	0.0014	6.65	13.61	12.71	5.81	6.24	11.59	5.45	7.43	4.34	14.09	6.73	Yolk
6	0.0015	8.50	14.73	14.15	6.95	8.37	10.79	5.68	6.98	4.12	15.10	4.63	Yolk
7	0.0016	3.14	16.01	7.22	8.28	10.64	7.68	8.31	7.34	6.10	15.30	9.97	Yolk
8	0.0017	8.97	12.95	13.89	6.78	8.01	10.23	5.75	7.76	3.89	14.79	6.98	Yolk
9	10.20	2.14	2.90	9.10	1.48	33.53	9.09	13.18	1.59	1.20	3.61	4.15	Glue
10	10.19	5.15	6.57	7.73	1.69	38.55	11.62	13.23	0.35	1.91	1.97	1.04	Glue
11	8.84	6.15	5.89	7.28	1.79	40.33	11.89	11.06	1.24	2.50	2.04	0.99	Glue
12	12.36	1.99	5.47	3.92	1.91	30.39	13.18	7.10	3.05	7.46	9.19	3.97	Glue
13	8.09	6.09	4.81	6.74	1.87	27.27	9.1	9.27	0.001	1.65	0.006	3.51	Glue
14	8.69	4.39	5.60	6.59	1.37	32.88	9.91	11.28	0.005	1.63	1.68	6.02	Glue
15	18.33	1.54	2.86	2.79	1.44	29.84	7.98	17.34	2.13	1.36	3.22	3.15	Glue
16	15.24	1.40	3.41	2.81	1.67	33.71	7.37	18.14	2.16	1.26	3.90	2.17	Glue
17	17.43	1.45	3.23	2.30	1.95	33.36	7.03	13.45	2.25	4.01	0.49	3.14	Glue
18	13.31	1.35	3.80	3.25	1.75	38.62	8.24	16.00	1.96	1.31	3.06	3.09	Glue
19	13.79	2.19	4.41	8.76	1.92	34.64	8.48	13.43	1.43	1.33	4.78	2.94	Fish
20	10.12	3.70	5.23	5.83	2.27	35.13	11.30	10.66	1.37	1.25	1.94	3.86	Fish

Table A1: *Training Set Data and Group Classifications for Standard Proteinaceous Media*

Case Name	OH-Pro	Ser	Asx	Glx	Arg/Thr	Gly	Ala	Pro	Val	Phe	Leu/Ile	Lys	Group
21	9.11	3.80	5.52	6.25	2.40	34.41	11.93	9.83	1.38	1.14	2.53	4.16	Fish
22	17.18	1.51	4.26	4.00	1.89	39.49	4.62	17.99	1.87	0.85	1.06	1.41	Fish
23	8.59	3.54	5.25	12.24	2.34	33.30	11.61	9.53	1.79	1.44	1.89	4.39	Fish
24	0.0018	1.80	9.85	11.03	3.46	8.37	5.90	7.07	9.73	8.12	18.62	6.64	Milk
25	0.0019	1.14	9.94	10.42	2.97	11.25	7.07	9.94	8.69	11.48	15.18	4.19	Milk
26	0.0001	3.64	9.97	15.91	3.74	6.44	4.93	5.65	10.03	6.15	19.78	4.99	Milk
27	0.0002	4.8	9.41	18.81	3.46	5.77	4.41	5.05	9.27	5.38	19.04	5.46	Milk
28	0.0003	4.17	9.83	17.34	3.40	6.20	4.69	5.56	9.56	6.91	19.52	3.56	Milk
29	0.0004	1.68	10.22	11.05	3.34	9.74	6.43	8.24	9.70	9.36	18.62	3.78	Milk
30	0.0005	1.10	9.46	10.58	2.89	10.84	7.05	9.48	8.35	11.46	14.04	7.21	Milk
31	25.45	0.69	1.36	2.09	1.32	31.23	2.53	27.15	0.36	1.01	1.34	0.42	Strong
32	25.15	0.75	1.51	2.16	1.45	31.37	2.67	25.39	0.41	0.97	1.25	0.51	Strong
33	31.58	0.59	1.18	1.67	0.78	28.61	2.10	28.54	0.34	0.64	0.33	0.0001	Strong
34	38.38	0.63	1.26	1.97	0.001	29.98	1.80	23.48	0.0001	0.0005	0.005	0.001	Strong
35	34.00	0.57	1.13	1.80	1.31	28.04	1.91	27.24	0.30	0.40	0.001	0.005	Strong
36	23.48	0.77	1.55	2.28	1.44	33.06	2.97	25.72	0.41	1.09	1.49	0.52	Strong
37	33.10	0.62	1.23	2.13	1.28	26.98	1.75	25.55	0.0005	0.33	0.0001	0.001	Skin
38	38.97	0.53	1.05	2.45	0.0001	30.42	1.88	19.47	0.001	0.005	0.001	0.0005	Skin
39	40.20	1.12	2.23	2.49	1.32	27.19	1.64	17.42	0.0001	0.001	0.005	0.005	Skin
40	18.80	1.07	2.14	2.81	1.70	35.46	3.94	23.07	0.46	1.53	2.39	1.22	Skin

Table A1: *Training Set Data and Group Classifications for Standard Proteinaceous Media*

Case Name	OH-Pro	Ser	Asx	Glx	Arg/Thr	Gly	Ala	Pro	Val	Phe	Leu/Ile	Lys	Group
41	24.91	1.06	2.11	2.39	1.42	30.22	2.66	27.17	0.41	1.08	1.69	0.58	Skin
42	25.87	1.02	2.04	1.98	1.35	29.73	2.56	27.35	0.39	1.01	1.54	0.47	Skin
43	21.90	1.12	2.24	4.08	2.58	22.31	4.86	27.49	1.85	0.43	2.96	1.76	Bone
44	15.57	0.93	1.86	2.74	1.64	36.14	5.87	20.54	0.41	2.11	3.14	2.88	Bone
45	28.53	0.75	1.50	2.78	1.56	28.54	3.09	24.90	0.31	0.95	0.53	0.69	Bone
46	21.04	1.29	2.57	3.71	1.65	34.83	3.77	12.32	1.81	2.00	4.17	2.26	Bone
47	13.97	1.17	2.33	4.52	2.04	38.44	5.27	17.70	0.52	2.04	2.74	2.41	Bone
48	10.70	1.46	2.93	5.18	1.63	36.15	8.94	13.87	0.55	2.65	3.90	3.61	Bone
49	0.0006	1.35	7.84	8.35	5.51	4.54	4.66	18.19	2.90	8.08	16.12	7.54	Casein
50	0.0007	0.99	6.95	5.82	7.00	5.27	5.92	20.63	2.93	7.33	13.62	4.73	Casein
51	0.0008	1.25	7.49	5.97	7.53	5.86	5.96	31.56	3.20	6.13	8.68	0.54	Casein
52	0.0009	1.29	7.74	5.89	7.17	5.81	5.92	27.07	3.45	6.72	10.24	1.41	Casein
53	0.001	0.60	5.78	3.92	6.90	5.60	5.62	39.09	2.61	4.61	6.65	1.07	Casein
54	0.0011	0.64	4.71	3.51	5.12	3.45	5.46	37.57	3.24	5.76	11.47	1.87	Casein
55	0.0011	3.68	7.36	3.66	8.47	11.26	4.47	12.56	4.54	7.33	11.03	4.06	Albumin
56	0.0012	3.34	6.69	3.46	8.19	11.50	3.82	15.37	4.44	5.78	7.89	1.50	Albumin
57	0.0013	1.03	9.34	3.26	8.36	11.63	4.19	14.64	4.41	6.95	8.85	1.12	Albumin
58	0.0014	3.37	6.74	3.26	7.84	10.58	3.98	14.49	4.33	7.32	10.65	1.51	Albumin
59	0.0015	4.92	10.53	9.45	5.21	7.61	9.27	4.96	3.81	8.58	15.05	5.03	Albumin
60	0.0016	3.31	9.87	7.05	5.27	8.00	8.03	5.39	4.36	8.75	15.37	6.66	Albumin

Table A1: *Training Set Data and Group Classifications for Standard Proteinaceous Media*

Case Name	OH-Pro	Ser	Asx	Glx	Arg/Thr	Gly	Ala	Pro	Val	Phe	Leu/Ile	Lys	Group
61	31.98	0.71	1.41	4.44	1.13	28.76	2.14	24.28	0.41	0.39	0.32	.0.75	Gelatine
62	36.99	0.77	1.53	5.33	1.40	29.56	1.77	17.25	0.0001	0.36	0.84	0.84	Gelatine
63	19.33	1.08	2.16	3.45	1.81	34.49	3.60	24.05	0.41	1.41	1.93	1.20	Gelatine
64	36.45	0.81	1.63	4.38	1.46	38.82	1.94	20.67	0.21	0.38	0.19	0.17	Gelatine
65	22.11	0.79	1.59	3.30	2.06	21.95	5.81	30.37	0.34	1.75	2.86	2.64	Gelatine
66	26.07	0.69	1.38	3.19	1.47	29.48	2.88	26.98	0.36	1.18	1.83	0.62	Gelatine
67	19.37	0.58	2.79	2.10	2.25	25.82	3.78	22.09	0.36	1.28	1.62	0.35	Parchment
68	32.42	0.42	2.18	1:65	1.80	27.89	7.07	22.79	0.17	0.38	0.32	0.0005	Parchment
69	24.99	0.40	2.23	1.71	1.93	30.13	2.82	25.29	0.21	0.98	1.09	0.38	Parchment
70	9.80	1.15	4.73	3.09	2.40	38.52	7.37	11.33	0.48	2.46	2.52	3.62	Sturgeon
71	13.02	2.86	7.80	6.38	3.78	44.44	2.96	2.96	0.63	0.52	1.05	1.63	Sturgeon
72	4.78	0.87	2.81	2.42	1.10	20.05	5.29	5.63	9.88	1.11	3.53	4.24	Sturgeon

Table A1: *Training Set Data and Group Classifications for Standard Proteinaceous Media*

Variable	Egg p=0.11111	Glue p=0.13889	Fish p=0.06944	Milk p=0.09722	Strong p=0.08333	Skin p=0.08333	Bone p=0.08333	Casein p=0.08333	Albumin p=0.08333	Gelatine p=0.08333	Parchment p=0.04167	Sturgeon p=0.04167
OH-Pro	9.642	7.140	7.200	8.401	7.734	7.686	7.206	7.123	7.283	7.773	7.172	6.549.
Ser	31.423	20.759	19.468	23.700	19.798	19.612	18.896	19.942	23.407	19.144	17.763	18.538
Asx	29.558	14.060	15.005	22.128	11.840	11.993	11.020	15.174	15.485	11.400	11.515	15.499
Glx	8.437	6.877	8.013	11.533	7.332	7.122	7.485	9.178	6.869	8.249	6.733	6.000
Arg/Thr	28.341	11.718	12.685	19.809	10.497	10.852	12.678	25.708	27.851	12.649	12.586	11.507
Gly	8.404	8.884	9.167	7.972	8.583	8.428	8.635	5.397	5.933	8.405	7.824	8.817
Ala	0.194	0.848	0.968	-0.389	0.238	0.236	0.577	0.128	0.043	0.326	1.353	0.528
Pro	13.214	9.506	9.664	12.160	10.121	9.831	9.531	11.036	9.690	10.080	9.304	8.399
Val	46.954	33.340	34.534	42.005	30.503	30.154	30.578	28.080	31.133	30.255	28.369	34.622
Phe	12.056	8.737	7.654	15.318	9.520	9.263	8.955	14.273	14.133	9.937	8.041	5.781
Leu/Ile	2.325	0.958	0.486	3.214	2.043	1.964	2.000	4.240	3.583	1.977	1.866	-0.004
Lys	23.834	16.269	17.114	17.527	15.264	15.131	15.512	14.894	15.115	15.530	14.514	16.193
Constant	-858.348	-416.103	-441.349	-663.665	-418.563	-404.898	-396.189	-481.471	-473.785	-418.441	-367.073	-387.464

Table A2: *Regression Weights and Constants for Variables in Each Group*

Root	Group											
	Egg	Glue	Fish	Milk	Strong	Skin	Bone	Casein	Albumin	Gelatine	Parchment	Sturgeon
1	-14.143	4.633	4.320	-8.918	7.005	6.807	6.085	-6.384	-7.166	6.129	6.387	4.540
2	-5.431	-2.297	-3.298	-0.708	0.532	0.502	0.213	8.241	5.329	1.030	1.455	-3.861
3	-1.976	-0.865	-0.647	5.959	1.033	0.586	0.014	-0.342	-3.090	1.050	-1.218	-1.953
4	-2.476	1.576	1.672	1.803	-2.341	-2.009	0.263	-0.539	2.162	-2.075	-0.722	4.158
5	-0.104	-0.324	-1.587	0.302	0.753	1.088	-0.197	-2.012	2.018	0.259	-0.926	0.404
6	-0.059	1.001	-0.087	0.031	-0.077	-0.319	0.205	-0.327	0.327	-0.145	-0.094	-2.340
7	0.025	-0.206	0.464	-0.207	-0.065	-0.237	0.850	0.100	0.081	0.535	-2.099	-0.099
8	0.011	0.485	-0.633	-0.096	0.546	0.247	-0.214	0.469	-0.344	-0.643	-0.964	0.477
9	-0.068	0.102	0.395	-0.032	-0.040	0.172	-0.740	0.052	0.077	0.314	-0.344	-0.068
10	0.013	0.081	-0.208	-0.003	-0.205	0.019	0.010	0.025	-0.054	0.183	0.001	0.093
11	-0.001	-0.018	0.057	0.003	-0.142	0.228	0.046	0.011	-0.009	-0.106	-0.030	-0.064

Table A3: *Means of Canonical Roots for Training Set*

Variable	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6	Test 7	Test 8	Test 9	Test 10
OH-Pro	8.84	10.19	12.36	0.50	15.67	29.44	16.32	0.0001	0.0001	0.0001
Ser	6.15	5.15	1.99	1.17	0.62	0.41	0.60	1.68	9.26	1.03
Asx	5.89	6.57	5.47	7.28	3.99	2.52	5.00	10.22	12.08	9.34
Glx	7.28	7.73	3.92	4.13	2.51	1.42	2.39	11.05	14.99	3.26
Arg/Thr	1.79	1.69	1.91	9.92	5.70	4.69	7.20	3.34	5.88	8.36
Gly	40.33	38.55	30.39	7.78	22.05	24.84	21.03	9.74	6.95	11.63
Ala	11.89	11.62	13.18	3.14	3.26	2.46	2.58	6.43	8.07	4.19
Pro	11.06	1323	7.10	17.32	23.13	23.71	28.21	8.24	5.76	14.64
Val	1.24	0.35	3.05	3.43	1.47	0.81	1.61	9.70	6.48	4.41
Phe	2.50	1.91	7.46	7.76	3.46	1.00	2.39	9.36	4.25	6.95
Leu/Ile	2.04	1.97	9.19	12.86	7.21	2.01	3.11	18.62	13.09	8.85
Lys	0.99	1.04	3.97	3.63	2.12	0.44	0.34	3.78	11.69	1.12

Table A4: *Data for Discriminant Analysis of Test Samples of Pure and Mixed Proteinaceous Media*

Test Sample	Sample Identity	Test Sample	Sample Identity
1	Animal glue adhesive	6	Egg albumin and animal glue
2	Animal glue adhesive	7	Egg albumin, casein and glue
3	Animal glue adhesive	8	Casein
4	Egg albumin and casein	9	Egg yolk
5	Casein and animal glue	10	Egg albumin

Test	Egg	Glue	Fish	Milk	Strong	Skin	Bone	Casein	Albumin	Gelatine	Parchment	Sturgeon
Test 1	318.292	**483.291**	**479.437**	371.772	**455.093**	**403.789**	**466.079**	341.485	368.787	**453.949**	**452.925**	**468.172**
Test 2	286.744	**454.159**	**452.193**	344.201	**430.503**	371.938	**439.799**	324.379	340.961	**430.171**	**430.946**	**439.538**
Test 3	280.328	**428.616**	**418.895**	356.404	**408.449**	363.466	**422.038**	348.609	370.629	**412.107**	**413.284**	**408.164**
Test 4	380.290	348.712	341.154	**412.805**	332.075	338.341	356.898	**498.017**	**506.152**	354.067	356.436	327.330
Test 5	282.489	**387.972**	**381.682**	**343.676**	**389.379**	**391.989**	**399.441**	**400.119**	400.264	**399.100**	**396.790**	**364.745**
Test 6	245.567	**395.742**	**390.641**	307.316	**405.528**	**407.968**	**408.023**	356.382	**360.055**	**411.840**	**408.392**	**374.880**
Test 7	359.621	**424.235**	**422.468**	**398.362**	**421.871**	**424.547**	**431.101**	**452.371**	**451.908**	**433.532**	**431.043**	**403.995**
Test 8	578.082	532.418	531.124	**648.168**	501.033	503.137	515.886	**579.938**	588.756	512.648	498.511	516.373
Test 9	**883.446**	697.201	704.810	89.024	642.591	646.616	661.945	749.576	779.552	659.390	642.434	681.109
Test 10	344.657	337.474	332.128	379.506	308.920	315.876	331.127	427.740	**445.384**	324.456	332.577	330.827

Table A5: *Classification Scores for Test Samples*

Work	OH-Pro	Ser	Asx	Glx	Arg/Thr	Gly	Ala	Pro	Val	Phe	Leu/Ile	Lys
N02880	1.69	4.45	10.15	32.05	3.47	8.93	6.96	14.23	1.06	4.59	5.22	3.92
N05500	2.09	4.67	11.15	25.82	4.21	12.49	10.54	8.68	3.10	7.54	3.60	1.22
N03059	6.07	9.39	19.05	12.03	5.24	29.54	11.95	6.74	0.001	0.00025	0.0075	0.0025
Ketel - Alice	7.34	3.30	12.71	7.94	3.12	33.54	9.76	10.56	6.49	5.25	0.0001	0.001
Ketel - Robert	4.36	4.11	24.63	4.25	3.64	26.41	9.87	7.10	9.04	2.60	0.00075	0.0075
Ketel - Joan	2.26	3.93	26.55	4.05	5.42	25.67	10.32	5.52	5.30	5.25	0.005	0.00075
N03545	5.79	7.72	9.47	15.47	9.07	21.59	8.42	11.08	2.43	0.0005	10.23	3.03
N01749	3.31	2.83	17.00	12.58	5.83	27.22	11.73	6.94	0.0005	0.005	0.0025	0.00025

Table A6: *Experimental Data for Suspected Mixed Proteinaceous Media Samples*

Work	Egg	Glue	Fish	Strong	Skin	Bone	Casein / Milk	Albumin	Gelatine	Parchment	Sturgeon
N02880	449.180	456.845	478.065	442.270	344.270	457.332	**538.610 / 560.804**	**483.647**	473.511	445.732	424.260
N05500	478.571	474.369	488.197	445.212	337.411	461.725	**533.593 / 572.163**	**511.095**	471.247	451.834	448.225
N03059	**657.564**	571.563	**582.200**	508.325	259.634	518.243	519.406	555.736	511.965	520.220	**578.317**
Ketel – Alice	**646.569**	643.567	**652.944**	590.025	464.486	599.488	538.557	573.170	591.714	583.489	646.947
Ketel – Robert	**961.546**	753.247	**772.344**	659.015	407.115	663.515	651.088	710.356	652.235	658.763	**792.243**
Ketel – Joan	**870.332**	658.138	**672.901**	567.979	297.270	575.293	616.947	670.028	566.303	578.907	**690.360**
N03545	**654.225**	575.703	585.635	539.939	453.169	565.215	**629.200 / 627.215**	**648.534**	561.353	554.353	558.556
N01749	**367.179**	**377.935**	**395.429**	324.511	158.375	344.580	346.934 / 364.896	357.940	335.418	354.241	**397.256**

Table A7: *Classification Scores for Suspected Mixed Proteinaceous Media Samples*

Statistical Information – Successive Roots Removed				Discriminant Function Analysis Summary			
Roots Removed	Eigenvalue	Wilks' Lambda	p-level	Variable	Wilks' Lambda	Partial Lambda	p-level
0	70.35806	0.000003	0.000000	OH-Pro	0.000009	0.352824	0.000000
1	16.42411	0.000218	0.000000	Ser	0.000007	0.459395	0.000021
2	6.30614	0.003803	0.000000	Asx	0.000007	0.420160	0.000003
3	4.61980	0.027786	0.000000	Glx	0.000006	0.484405	0.000064
4	1.28818	0.156149	0.000026	Arg/Thr	0.000007	0.463681	0.000026
5	0.48106	0.357297	0.030841	Gly	0.000007	0.445518	0.000011
6	0.35957	0.529177	0.161815	Ala	0.000005	0.635190	0.012069
7	0.24712	0.719452	0.494296	Pro	0.000009	0.323305	0.000000
8	0.09014	0.897240	0.894716	Val	0.000010	0.300941	0.000000
9	0.01312	0.978115	0.971346	Phe	0.000006	0.548542	0.000781
10	0.00914	0.990946	0.764677	Leu/Ile	0.000004	0.770950	0.240386
-	-	-	-	Lys	0.000007	0.448349	0.000013

Table A8: *Discriminant Analysis – Statistical Summary*

Eigenvalues - represent the amount of discrimination achieved as a percentage. Values above 1 are significant, values below 1 are due to random noise/variation: 4 roots are important in the above case. **Wilks' Lambda/Partial Lambda** – the smaller the value, the greater the importance of the root/variable to discrimination. **p-level** – represents the statistical significance of the root/variable to discrimination between the groups. The lower the value, the greater the significance.

Variable (retn. time / min)	Gum											
	Arabic Replicates						Tragacanth Replicates					
1 (12.57)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
2 (13.24)	0.649	0.275	0.359	0.424	0.375	0.689	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
3 (13.78)	2.689	1.294	1.793	2.098	2.094	1.175	2.902	1.150	0.404	0.796	0.753	2.585
4 (14.08)	1.068	0.468	0.543	0.522	0.917	0.0001	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
5 (14.33)	1.135	0.725	0.924	0.837	0.896	0.796	0.981	0.496	0.172	0.423	0.382	1.207
6 (14.39)	1.108	0.716	0.946	0.891	0.927	0.544	1.240	0.554	0.183	0.473	0.417	1.390
7 (14.65)	1.284	0.908	1.109	0.924	1.094	0.641	1.376	0.621	0.226	0.554	0.510	1.536
8 (14.79)	1.514	1.670	1.054	1.185	1.302	0.592	1.676	0.645	0.221	0.483	0.417	1.366
9 (15.40)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
10 (15.78)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.777	0.422	0.194	0.353	0.313	0.878
11 (16.09)	1.311	0.917	1.120	1.054	1.188	0.699	0.981	0.364	0.172	0.353	0.347	1.000
12 (16.18)	1.243	1.009	1.326	1.359	1.167	2.058	1.499	0.885	0.652	1.058	1.251	2.122
13 (16.28)	1.743	1.404	1.511	1.446	1.531	1.369	1.935	1.406	0.916	1.662	1.818	2.085
14 (16.40)	0.527	0.257	0.391	0.467	0.542	0.0001	0.313	0.0001	0.0010	0.0001	0.0010	0.256
15 (16.52)	0.635	0.560	0.587	0.565	0.531	1.825	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
16 (16.73)	0.878	0.541	0.696	0.663	0.771	0.689	0.981	0.347	0.178	0.312	0.313	0.963
17 (16.90)	1.986	1.945	1.989	1.728	1.896	1.699	2.017	1.754	1.147	2.145	2.467	2.438
18 (17.11)	1.324	0.908	1.076	1.043	1.135	0.874	0.409	0.0001	0.108	0.0010	0.0001	0.500
19 (17.20)	0.716	0.330	0.478	0.533	0.667	0.0001	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
20 (17.30)	1.473	1.303	1.652	1.652	1.688	0.485	0.504	0.273	0.0001	0.292	0.255	0.658

Table A9: *Training Set Data and Group Classification for Standard Gum Media*

Variable (retn. time / min)	Gum											
	Arabic Replicates						Tragacanth Replicates					
21 (17.43)	0.500	0.440	0.533	0.511	0.490	0.835	0.722	0.447	0.307	0.514	0.591	0.951
22 (17.55)	0.365	0.193	0.228	0.0001	0.333	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
23 (17.71)	1.162	0.853	1.022	1.054	1.135	0.621	0.313	0.0001	0.108	0.0010	0.151	0.378
24 (17.84)	0.473	0.394	0.446	0.489	0.417	0.777	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
25 (18.01)	1.054	0.624	0.859	0.891	0.854	0.718	0.232	0.0001	0.0010	0.0001	0.0010	0.0001
26 (18.12)	0.284	0.0001	0.141	0.0010	0.281	0.0001	0.300	0.356	0.269	0.423	0.452	0.378
27 (18.19)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
28 (18.34)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
29 (18.46)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
30 (18.84)	2.878	1.853	2.315	2.304	2.219	1.515	0.654	0.306	0.172	0.222	0.174	0.597
31 (18.98)	0.378	0.275	0.359	0.337	0.313	0.0001	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
32 (19.10)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.300	0.390	0.291	0.473	0.498	0.378
33 (19.45)	0.554	0.394	0.543	0.478	0.479	0.447	0.286	0.0001	0.0010	0.0001	0.0010	0.305
34 (19.63)	0.459	0.321	0.446	0.402	0.365	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
35 (19.71)	2.135	1.661	1.815	1.641	1.729	1.068	0.531	0.281	0.162	0.222	0.162	0.537
36 (19.99)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.259	0.0001	0.081	0.101	0.0010	0.0001
37 (20.16)	0.419	0.275	0.380	0.446	0.458	0.592	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
38 (20.25)	0.284	0.275	0.283	0.261	0.260	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
39 (20.35)	0.986	0.688	0.913	0.935	1.073	0.767	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
40 (20.97)	1.000	1.000	1.000	1.000	1.000	1.000	0.232	0.232	0.232	0.232	0.232	0.232

Table A9: *Training Set Data and Group Classifications for Standard Gum Media*

Variable (retn. time / min)	Gum											
	Arabic Replicates						Tragacanth Replicates					
41 (21.57)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
42 (21.75)	1.608	1.477	1.511	1.467	1.354	1.136	0.313	0.389	0.377	0.373	0.637	0.317
43 (23.06)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
44 (28.15)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
45 (28.22)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010

Variable (retn. time / min)	Gum											
	Cherry Replicates						Karaya Replicates					
1 (12.57)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.100	0.056	0.0001	0.401	0.187	0.095
2 (13.24)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.223	0.128	0.197	0.202	0.431	0.202
3 (13.78)	0.459	2.242	0.543	0.281	0.116	0.022	0.162	0.087	0.148	0.130	0.262	0.125
4 (14.08)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.190	0.102	0.129	0.111	0.197	0.077
5 (14.33)	0.186	0.468	0.334	0.178	0.077	0.0001	0.318	0.194	0.302	0.293	0.571	0.285
6 (14.39)	0.230	0.860	0.348	0.185	0.082	0.0001	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
7 (14.65)	0.247	1.077	0.439	0.240	0.101	0.0001	0.067	0.036	0.062	0.058	0.103	0.053
8 (14.79)	0.273	1.230	0.313	0.157	0.063	0.253	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
9 (15.40)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.067	0.046	0.068	0.072	0.112	0.071
10 (15.78)	0.100	0.533	0.230	0.137	0.063	0.150	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
11 (16.09)	0.286	0.805	0.355	0.233	0.096	0.075	0.425	0.312	0.370	0.391	0.655	0.350
12 (16.18)	0.884	1.415	0.717	0.582	0.439	0.338	0.117	0.092	0.123	0.124	0.159	0.107

Table A9: *Training Set Data and Group Classifications for Standard Gum Media*

Variable (retn. time / min)	Gum											
	Cherry Replicates						Karaya Replicates					
13 (16.28)	0.511	1.916	1.211	1.041	0.748	0.790	0.072	0.061	0.080	0.072	0.066	0.071
14 (16.40)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.095	0.076	0.117	0.137	0.384	0.160
15 (16.52)	0.139	0250	0.063	0.082	0.053	0.038	0.536	0.409	0.480	0.437	0.496	0.397
16 (16.73)	0.269	0.631	0.230	0.130	0.077	0.0001	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
17 (16.90)	0.559	2.198	1.482	1.465	1.013	1.155	0.190	0.138	0.185	0.202	0.271	0.190
18 (17.11)	0.307	0.500	0.445	0.315	0.135	0.177	0.827	0.659	0.911	0.873	1.395	0.695
19 (17.20)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
20 (17.30)	0.346	0.784	0.522	0.390	0.149	0.236	1.156	1.018	1.212	1.362	1.994	1.234
21 (17.43)	0.390	0.566	0.320	0.267	0.212	0.188	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
22 (17.55)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
23 (17.71)	0.511	0.490	0.341	0.240	0.125	0.124	0.463	0.384	0.468	0.495	0.711	0.451
24 (17.84)	0.156	0.0010	0.090	0.055	0.043	0.0001	0.302	0.261	0.332	0.346	0.618	0.368
25 (18.01)	0.464	0.373	0.209	0.110	0.067	0.0001	0.400	0.302	0.431	0.449	0.599	0.415
26 (18.12)	0.087	0.370	0.292	0.287	0.198	0.215	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
27 (18.19)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.084	0.076	0.092	0.104	0.0001	0.095
28 (18.34)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
29 (18.46)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
30 (18.84)	0.758	1.001	0.543	0.287	0.169	0.032	1.145	0.885	1.305	1.349	1.835	1.240

Table A9: *Training Set Data and Group Classifications for Standard Gum Media*

Variable (retn. time / min)	Gum											
	Cherry Replicates						Karaya Replicates					
31 (18.98)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.179	0.158	0.228	0.248	0.412	0.249
32 (19.10)	0.056	0.305	0.306	0.329	0.222	0.263	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
33 (19.45)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.229	0.179	0.259	0.319	0.496	0.320
34 (19.63)	0.156	0.283	0.070	0.0001	0.0010	0.075	0.156	0.123	0.179	0.209	0.318	0.219
35 (19.71)	0.529	0.0001	0.529	0.287	0.164	0.032	1.095	0.823	1.206	1.264	1.545	1.027
36 (19.99)	0.117	0.141	0.042	0.041	0.029	0.038	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
37 (20.16)	0.286	0.0001	0.049	0.028	0.029	0.0010	0.106	0.082	0.129	0.169	0.281	0.178
38 (20.25)	0.139	0.0001	0.125	0.130	0.130	0.113	0.184	0.179	0.179	0.183	0.169	0.166
39 (20.35)	0.394	0.207	0.100	0.055	0.048	0.048	0.190	0.154	0.221	0.300	0.440	0.279
40 (20.97)	0.424	0.424	0.424	0.424	0.424	0.424	0.665	0.665	0.665	0.665	0.665	0.665
41 (21.57)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
42 (21.75)	0.511	0.577	0.710	0.835	0.709	0.661	1.034	1.089	1.071	1.108	0.908	0.979
43 (23.06)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
44 (28.15)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
45 (28.22)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010

Table A9: *Training Set Data and Group Classifications for Standard Gum Media*

Variable (retn. time / min)	Gum											
	Ghatti Replicates						Guar Replicates			Locust Bean Replicates		
1 (12.57)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
2 (13.24)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
3 (13.78)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
4 (14.08)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
5 (14.33)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
6 (14.39)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
7 (14.65)	0.121	0.142	0.0001	0.154	0.0010	0.070	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
8 (14.79)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
9 (15.40)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
10 (15.78)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
11 (16.09)	0.181	0.226	0.090	0.205	0.0001	0.083	0.038	0.141	0.111	0.037	0.045	0.024
12 (16.18)	1.049	1.271	0.956	1.284	2.296	0.892	0.038	0.057	0.062	0.017	0.019	0.016
13 (16.28)	4.257	4.633	3.786	4.442	4.483	1.338	0.076	0.084	0.086	0.056	0.036	0.034
14 (16.40)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
15 (16.52)	0.263	0.226	0.307	0.257	0.957	0.255	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
16 (16.73)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
17 (16.90)	4.035	5.932	3.751	5.932	3.751	5.393	0.126	0.141	0.136	0.066	0.058	0.047
18 (17.11)	0.343	0.396	0.198	0.334	0.437	0.204	0.277	0.339	0.300	0.132	0.120	0.079
19 (17.20)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
20 (17.30)	0.565	0.763	0.180	0.668	0.0001	0.357	0.428	0.509	0.445	0.185	0.219	0.105

Table A9: *Training Set Data and Group Classifications for Standard Gum Media*

Variable (retn. time / min)	Gum											
	Ghatti Replicates						Guar Replicates			Locust Bean Replicates		
21 (17.43)	0.646	0.735	0.595	0.693	1.093	0.612	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
22 (17.55)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.717	0.720	0.667	0.371	0.297	0.176
23 (17.71)	0.222	0.254	0.126	0.231	0.464	0.147	0.176	0.212	0.185	0.095	0.094	0.052
24 (17.84)	0.060	0.0001	0.072	0.0010	0.0001	0.083	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
25 (18.01)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.100	0.113	0.148	0.024	0.045	0.016
26 (18.12)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
27 (18.19)	0.263	0.254	0.270	0.282	0.629	0.230	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
28 (18.34)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.252	0.226	0.272	0.0001	0.084	0.0010
29 (18.46)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.038	0.042	0.049	0.022	0.019	0.0001
30 (18.84)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.063	0.042	0.074	0.0001	0.019	0.0010
31 (18.98)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
32 (19.10)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.029	0.026	0.018
33 (19.45)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
34 (19.63)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.034	0.016	0.031

Table A9: *Training Set Data and Group Classifications for Standard Gum Media*

Variable (retn. time / min)	Gum											
	Ghatti Replicates						Guar Replicates			Locust Bean Replicates		
35 (19.71)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
36 (19.99)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	2.705	3.036	2.656	0.522	1.291	0.561
37 (20.16)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
38 (20.25)	0.242	0.282	0.253	0.282	0.246	0.204	0.176	0.183	0.185	0.093	0.094	0.073
39 (20.35)	0.121	0.113	0.0001	0.103	0.109	0.076	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
40 (20.97)	0.847	0.847	0.847	0.847	0.847	0.847	0.692	0.692	0.692	0.420	0.420	0.420
41 (21.57)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.113	0.127	0.111	0.071	0.071	0.058
42 (21.75)	1.574	1.553	1.352	1.541	1.258	1.280	2.088	1.948	1.792	0.447	0.694	0.538
43 (23.06)	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010	0.151	0.170	0.148	0.105	0.100	0.068
44 (28.15)	0.242	0.311	0.180	0.282	0.902	0.230	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010
45 (28.22)	0.181	0.142	0.144	0.180	0.765	0.223	0.0001	0.0010	0.0001	0.0010	0.0001	0.0010

Table A9: *Training Set Data and Group Classifications for Standard Gum Media*

Variable	Group						
	Arabic p=0.16667	Tragacanth p=0.16667	Cherry p=0.16667	Karaya p=0.16667	Ghatti p=0.16667	Guar p=0.08333	Locust Bean p=0.08333
2	-206.87	143.87	-35.76	-210.56	-84.32	34.39	42.42
3	67.18	-209.25	142.61	-345.38	24.06	-63.29	-45.03
4	-46.61	120.16	-53.19	9.63	10.42	70.30	57.75
5	112.81	55.41	-0.79	239.64	14.15	-99.96	-57.89
6	-428.96	501.74	-154.60	-85.58	-218.32	228.20	187.26
7	791.48	-936.80	251.55	248.03	259.56	-314.27	-281.80
8	-67.69	1.582	-37.11	-254.71	150.32	-30.95	12.91
10	-4186.95	2040.31	62.66	-386.12	-1993.40	-203.34	-246.29
11	-394.54	320.19	87.77	-134.31	-179.17	39.57	43.18
12	237.48	-90.24	-22.15	190.12	58.37	-13.72	-65.02
13	-475.60	307.42	-160.05	-140.88	-378.55	194.38	146.12
14	10.16	18.09	1.58	7.88	57.11	5.43	6.52
15	-352.98	-12.76	-4.81	-500.74	-96.41	40.17	14.29
16	394.24	-312.45	45.35	205.22	190.09	-106.70	-85.74

Table A10: *Regression Weights and Constants for Variables in Each Group*

Variable	Group						
	Arabic p=0.16667	Tragacanth p=0.16667	Cherry p=0.16667	Karaya p=0.16667	Ghatti p=0.16667	Guar p=0.08333	Locust Bean p=0.08333
17	-583.07	334.33	-9.97	-78.98	-551.21	-4.30	3.63
18	-8.64	-14.95	5.31	-6.49	-42.88	-9.74	-8.98
21	148.78	-90.03	12.30	113.65	40.57	-9.15	1.08
22	908.10	-422.49	285.96	250.68	883.00	-319.23	-235.50
23	-144.94	72.82	-34.93	-105.01	-85.23	241.78	160.95
24	133.99	-116.84	108.62	-20.11	140.53	-65.21	-38.42
29	391.26	-480.59	-9.31	204.95	110.89	-104.97	-437.27
34	893.03	-415.49	-16.29	649.91	450.49	-123.64	-75.80
37	-69.21	74.56	-0.39	-37.08	-19.07	57.64	63.64
Constant	-426.01	-313.08	-37.07	-208.06	-194.77	-216.17	-98.30

Table A10: *Regression Weights and Constants for Variables in Each Group*

The Analyst

Critical Review

Applications of Chromatography in Art Conservation: Techniques Used for the Analysis and Identification of Proteinaceous and Gum Binding Media

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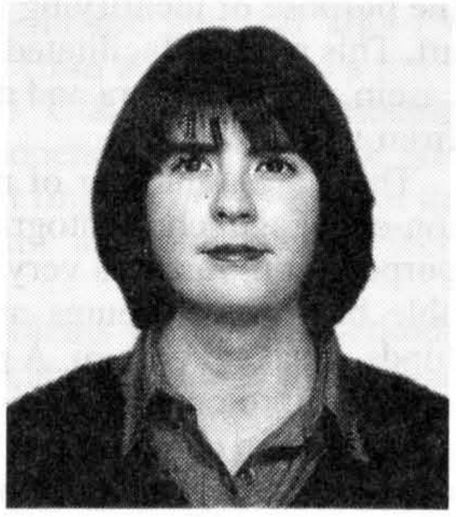
Summary of Contents

Keywords: *Gas chromatography; high-performance liquid chromatography; proteinaceous media; polysaccharide gums; art conservation; review*

Sarah Vallance graduated in 1992 with a BSc in Applied Chemistry from the University of Northumbria at Newcastle. After a period in industry, she returned to the university and recently completed her programme of research for the award of PhD. She is at present preparing her thesis, *The Development and Application of Chromatographic Methods in the Characterisation of Artists' Media*, for examination.



Introduction

There is a need for the development of accurate and reliable methods for the analysis of samples from works of art in order to meet the specific requirements of the conservator. A detailed awareness of the constituents of paint layers from easel paintings, for example, provides the conservator with the background information required to facilitate the design of the optimum safe conservation/restoration treatment plan, taking into account the nature of the original materials used.

From a practical aspect, specific knowledge of the nature of the media in particular works may offer some indication as to why some paintings are in better condition than others of a similar age. This type of information also enables the art historian to come to an educated and informed conclusion regarding the age and potential origin of an unknown work. In addition, it is feasible that a counterfeit work may be revealed, if the artist has been careless enough to use pigments or binders that are historically incorrect.

The most important factors in all the techniques to be discussed are the extensive methods of sample preparation deemed necessary, since the chromatographic techniques used in the analyses are not extraordinary. The microscopic samples characteristic of work in this area are notoriously problematic to deal with and sensitivity is paramount, which consequently places great importance on the laboratory skills of the conservation scientists themselves.

Paint Media

Since man first learned to paint, artists have used a diverse variety of binding media for their pigments, ranging from natural gums and oils to proteinaceous materials such as egg (glair and tempera), milk (casein) and collagen glues made from animal skins and skeletons, for example.

Chemically, oils and fats are the glycerol esters of aliphatic acids, typically those of the 18-carbon series.[1] Oils tend to be liquid at room temperature, whereas fats are solid or semi-solid and greasy to the touch. If an oil possesses sufficient di- and triunsaturated fatty acids in its triglyceride components it will 'dry', *i.e.*, polymerise, giving rise to a semi-solid. The drying oils most widely used in western European art are linseed (obtained from the seeds of the flax), walnut and poppy, although the time when they were first used for painting purposes is not known. Analytical evidence suggests that linseed oil was used in northern Europe from, at latest, the 13th Century, whilst in Italy, where oil painting was introduced in the 15th Century, walnut oil was initially preferred, although linseed oil became more common there from the 16th Century.[2] In most recent years, the use of other oils, such as safflower and tung, has become more common.[3–5]

Collagen[6] is the predominant proteinaceous material in animal skeletons (both skin and bone), representing one third of the total protein present in mammalian organisms. Collagen production in the body is preceded by the production of procollagen, a much larger biosynthetic precursor molecule, which is then degraded by specific enzymes resulting in collagen. There are a number of different types of collagen, but they all consist of molecules which contain three polypeptide α-chains in a triple helix conformation. Each α-chain has an amino acid sequence which is mainly a repeating structure, with glycine as every third residue and either proline or hydroxyproline often preceding the glycine residues. The various types of collagen can be distinguished by the slight differences in the sequence of their constituent amino acids.

Animal and fish collagen glues are widely used as strong adhesives for wood, binders in the preparation of grounds, size for canvas and pigment binders in decorative paints.[7] Preparation is relatively simple, involving the treatment of specific collagen-containing animal or fish tissues with hot water. When the leached solution cools, it forms a gelatinous mass; gelling occurs as a result of the partial decomposition of the tissues. If

present in the sample? 4. Have any conservation/restoration treatments been performed on the work previously? If so, what was the nature of any treatment, *e.g.*, consolidation, retouching?

The conservation scientist needs to select a technique which will give the maximum amount of information for the minimum amount of sample and sample preparation. For proteinaceous media, this would appear to be amino acid determination by RP-HPLC using FMOC as the derivatising agent. Gas chromatography of silylated sugar derivatives is, at present, probably the best method for the identification of natural gums: however, as this is probably the least investigated area so far, major developments in methodology which would greatly improve sensitivity can be expected; the main problem with samples of gum media is the minute amount of actual medium present.

Simple qualitative techniques such as TLC, microscopy and staining tests may be sufficient to indicate the basic media type used in a work, but as more and more works of art require conservation/restoration treatments it is crucial that the conservator has as much information as possible on the nature of any materials used by the artist, in order to avoid the loss or spoiling of any valuable and irreplaceable pieces.

It is clear that the previous investigations into quantitative analyses are of immense value and as chromatographic techniques are continually developed and improved, increasing both sensitivity and reproducibility, their use in the area of art conservation becomes even more ideal. One possibility is the development analytical methods using capillary zone electrophoresis (CZE), a relatively new technique currently used for the determination of proteins, *etc.*; it could prove to be a useful method of analysis in this area, owing to its high sensitivity, but a thorough investigation into its suitability would be required. Microbore techniques should also find their use in conservation science, since they obviously suit the sometimes nanomole amounts of samples which are provided for analysis.

The main concern of the conservation scientist is the availability of reliable and accurate analytical techniques suitable for use with the minute samples typically seen in this field of work. No doubt further research will result in the simplification of methods of sample preparation, possibly negating the need for clean-up procedures (*e.g.*, the removal of pigments from samples) prior to analysis. However, any improvements which mean that the required sample size is reduced and the loss of valuable sample material is minimised or, ideally, eliminated will be wholeheartedly welcomed by the conservator and conservation scientist alike.

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Paper 6/06219I
Received September 9, 1996
Accepted March 20, 1997